## REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

## I. CLAIM STATUS & AMENDMENTS

Claims 1-3 and 5 were pending in this application when last examined and stand rejected.

Claim 3 is amended to be directed to "a polynucleotide encoding a human protein hAMAH, which is a signal transduction molecule for cell proliferation, wherein the polynucleotide consists of the nucleotide sequence from the 11th to the 1285th nucleotide of SEQ ID No: 2." Support for this amendment can be found at page 3, lines 18-21, at page 4, lines 12-14, and SEQ ID Nos: 1-2. In this regard, a review of the disclosure and the Sequence Listing reveals that the nucleotide sequence of the 11th to the 1285th nucleotide of SEQ ID No: 2 is the coding region for the human protein hAMSH of SEQ ID No. 2. In other words, SEQ ID NO: 1 is a protein of 424 amino acids, which is encoded by the polynucleotide of the 11th to the 1285th nucleotide of SEQ ID No: 2. It is noted that the 424 amino acid hAMSH protein is encoded by a 1272 nucleotide sequence, which corresponds to the 1275 nucleotide sequence of 11th to the 1285th nucleotide of SEQ ID No: 2, minus a 3 nucleotide start/stop codon.

Claim 5 is amended to recite "polynucleotide" instead of "cDNA" to be consistent with the amendment to claim 3. Support can be found in the specification at the locations noted above.

Therefore, no new matter has been added by this amendment.

## II. INDEFINITENESS REJECTION

On page 2 of the Office Action, claims 1-3 and 5 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for "which is a signal transduction molecule for cell proliferation."

This rejection is respectfully traversed as applied to the amended claims.

The hAMSH protein is essential for increasing (or maintaining) cell proliferation. For instance, based on the disclosure at page 4, lines 11-24 and lines 21-23, it is evident that the claimed hAMSH protein is a novel molecule involved in signal transduction for cell proliferation by association with "STAM" downstream of the cytokine receptor. Also, at page 2, lines 18-24 of the disclosure, it is indicated that STAMs play an important role in the expression of c-myc and signal transduction for DNA synthesis, which leads to cell proliferation.

The specification discloses that AMSH-dc2, in which half of the C-terminal region of hAMSH as been deleted, suppresses signal transduction for DNA synthesis and c-myc inducing signal transduction after stimulating with IL-2 and GM-CSF (page 4, lines 25-29). This implies that the intact hAMSH has a role for maintaining signal transduction, and thus, the hAMSH protein is essential for cell proliferation.

In addition, for one of skill in the art, it has been well accepted that confirming "signal transduction for DNA synthesis and c-myc inducing signal transduction after stimulating with IL-2 and GM-CSF" is effective for identifying factors involved in a signal transduction pathway that is essential for cell proliferation. In this regard, see the attached copies of Shibuya al., Cell, vol. 70, pp. 57-67, 1992 , and Miyazaki et al., Cell, vol. 81, pp. 223-232, 1995.

Based on the above, it is respectfully submitted that one of skill in the art would clearly recognize and understand the metes and bounds of "signal transduction for cell proliferation."

Thus, the rejection of claims 1-3 and 5 under 35 U.S.C. § 112, second paragraph, is untenable and should be withdrawn.

## III. ANTICIPATION REJECTION

On page 3 of the Office Action, claims 2-3 were rejected under 35 U.S.C. § 102(b), as anticipated by Yu et al., NCBI Accession No. AF052135, August 5, 1998, hereinafter Yu (AF052135).

This rejection is respectfully traversed as applied to the amended claims.

To anticipate a claim, a cited prior art reference must either expressly or inherently teach each and every element of the claimed invention. See M.P.E.P. § 2131.01.

Amended claims 2-3 call for an isolated human gene encoding protein hAMSH of SEQ ID No: 1 and specific polynucleotide consisting of the 11$^{th}$ to 1285$^{th}$ nucleotide of SEQ ID No: 2.

As noted in the last response, Yu (AF052135) fails to disclose or suggest an hAMSH protein, let alone one having the amino acid sequence of SEQ ID No. 1. In fact, Yu (AF052135) fails to disclose an amino acid sequence. Instead, Yu (AF052135) discloses a nucleic acid, and not a protein. Also, Yu (AF052135) fails to disclose the function of the protein as a signal transduction molecule for cell proliferation.

Furthermore, Yu (AF052135) describes a cDNA clone that contains the 1st-1356th nucleotide sequence of SEQ ID No: 2 of the instant application. That is, the 107th-1462nd nucleotide sequence of Yu (AF052135) is identical to the 1st-1356th nucleotide sequence of SEQ ID No: 2.

However, Yu (AF052135) never discloses the coding region for a protein. The coding region starts a the "atg" (Met) codon, but Yu (AF052135) contains three candidates for the start codons (117-119; 127-129; 133-135). Therefore, one of skill in the art cannot ascertain the coding region of hAMSH from the disclosure of Yu (AF052135). Consequently, Yu (AF052135) fails to disclose or suggest the specific polynucleotide consisting of the nucleotide from the 11th to the 1285th nucleotide of SEQ ID No: 2 (the coding region for hAMSH) of the claims.

Therefore, Yu (AF052135) fails to disclose each and every element of the claimed invention. In view of the above, the rejection of claims 2-3 under 35 U.S.C. § 102(b) is untenable and should be withdrawn.

## IV. OBVIOUSNESS REJECTION

On page 4 of the Office Action, claim 5 was rejected under 35 U.S.C. § 103(a) as obvious over Yu (AF052135).

This rejection is respectfully traversed for the same reasons set forth immediately above. Therefore, the rejection of claim 5 under 35 U.S.C. § 103(a) is untenable and should be withdrawn.

## CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

Kazuo SUGAMURA et al.

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December 7, 2005

## ATTACHMENTS

1. Shibuya et al., "IL-2 and EGF Receptors Stimulate the Hematopoietic Cell Cycle via Different Signaling Pathways: Demonstration of a Novel Role for c-myc", Cell, Vol. 70, pp. 57-67, 1992; and

2. Miyazaki et al., "Three Distinct IL-2 Signaling Pathways Mediated by bcl-2, c-myc, and lck Cooperate in Hematopoietic Cell Proliferation", Cell, Vol. 81, pp. 223-231, 1995.

Cell, Vol. 70, 57–67, July 10, 1992, Copyright © 1992 by Cell Press

# IL-2 and EGF Receptors Stimulate the Hematopoietic Cell Cycle via Different Signaling Pathways: Demonstration of a Novel Role for c-*myc*

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## Summary

**Stimulation via cytokine receptors such as IL-2 and IL-3 receptors, but not by the EGF receptor (EGFR), induces cells of the BAF-B03 hematopoietic cell line to transit the cell cycle. We demonstrate that the IL-2 receptor β chain (IL-2Rβ) is linked to at least two intracellular signaling pathways. One pathway may involve a protein tyrosine kinase of the src family, which leads to the induction of the c-*jun* and c-*fos* genes, among others. A second pathway, involving an as yet unknown mechanism, leads to c-*myc* gene induction. Stimulation of the EGFR, expressed following transfection of an appropriate recombinant construct, can activate the former, but not the latter, pathway in this cell line and cause the cells to enter S phase but not progress further. This deficiency can be rescued by ectopic expression of the c-*myc* gene, indicating a novel role for this proto-oncogene in the S to G2/M transition of the cell cycle.**

## Introduction

Cytokines are the critical regulators of proliferation and differentiation for hematopoietic cells. Recent advances in the molecular characterization of cytokine receptor genes have revealed a new family of receptors that permits the cytokines to stimulate responses in target cells (D'Andrea et al., 1989; Bazan, 1990; Cosman et al., 1990; Hatakeyama, 1992). It is particularly important that members of this new receptor family lack the intrinsic protein tyrosine kinase domain that is the hallmark for the receptors that bind other growth factors such as epidermal growth factor (EGF), platelet-derived growth factor, and macrophage colony-stimulating factor (CSF-1) (Ullrich and Schlessinger, 1990; Sherr, 1991). Indeed, very little is known regarding the mechanism whereby cytokine receptors deliver signals to the cell interior.

The availability of cytokine receptor cDNAs has made it feasible to gain further insights into the coupling of receptors with putative intracellular components that evoke functional responses in host cells. It has become clear that the receptors of this new family, such as the interleukin 2 (IL-2) receptor β chain (IL-2Rβ), can mediate a proliferative signal in cultured hematopoietic cells but not in fibroblast cells (Doi et al., 1989a; Hatakeyama et al., 1989; Tsudo et al., 1989; Minamoto et al., 1990; Tanaka et al., 1991). Furthermore, the cytoplasmic region of the IL-2Rβ critical for signal transduction has been well identified by expressing IL-2Rβ mutant cDNAs in a hematopoietic cell line, BAF-B03 (Hatakeyama et al., 1989). We have recently shown that the IL-2Rβ chain interacts with a lymphocyte-specific nonreceptor protein tyrosine kinase, p56$^{lck}$ (Hatakeyama et al., 1991). In fact, IL-2 was found to induce the p56$^{lck}$ tyrosine kinase in peripheral blood lymphocytes (Hatakeyama et al., 1991; Horak et al., 1991). However, an IL-2Rβ mutant lacking the interaction site for p56$^{lck}$ in the cytoplasmic region retained the ability to induce mitogenic responses in the BAF-B03 cells (Hatakeyama et al., 1989, 1992). These observations suggest the existence of an IL-2R-mediated signaling pathway(s) that is distinct from protein tyrosine kinase activation but that is essential for the proliferation of hematopoietic cells. However, the nature and the target genes of such a signaling pathway(s) still remain elusive.

Evidence has accumulated for the importance of nuclear proto-oncogenes as potentially critical targets for proliferation signals delivered by growth factors (Cantley et al., 1991; Hunter, 1991). Most of the nuclear proto-oncogene products manifest DNA-binding properties either singly or in combination with others, thereby functioning as regulators of gene transcription (Gutman and Wasylyk, 1991). Nuclear proto-oncogene induction is followed and/or accompanied by progression through the cell cycle, wherein the expression of cell cycle regulators such as cyclins and cdc2 family kinases is induced. In mammalian cells, seven cyclin species (cyclin A, B, C, D1–D3, and E) and at least two cdc2 family kinases (cdc2 and cdk2) have been described, and their functional role during the cell cycle is beginning to emerge (Pines and Hunter, 1990; Lew et al., 1991; Matsushime et al., 1991; Lee and Nurse, 1987; Ninomiya-Tsuji et al., 1991; Elledge and Spottswood, 1991; Koff et al., 1991; Tsai et al., 1991). In brief, a number of evidence indicates that cyclins C, D, E, and cdk2 kinase function at the G1 to S phase transition, whereas cyclins A, B, and cdc2 kinase primarily act at the G2/M phase of the cell cycle. Cyclin A also appears to play a role in the G1 to S phase (Girard et al., 1991).

As an approach to gain further insights into signal transduction pathways and the critical target genes for cytokine receptors, we have analyzed the properties of IL-2 and EGF receptors (EGFRs) expressed in BAF-B03 cells, particularly with respect to their ability to induce expression of critical nuclear proto-oncogenes, the cyclins and cdc2 family kinases. The BAF-B03 cell line is a subline of BA/F3, a murine bone marrow–derived pro-B cell line whose viability and proliferation depend on interleukin-3 (IL-3) (Palacios and Steinmetz, 1985). Although the BA/F3 cell line was originally selected for IL-3 dependency, it can also be stimulated by numerous other cytokines when their

respective receptor cDNAs are expressed following cDNA transfection (Hatakeyama et al., 1989; Hibi et al., 1990; Li et al., 1990; Fukunaga et al., 1991).

Our experimental approach has allowed us to reveal the presence of at least two distinct receptor signaling pathways for proto-oncogene induction; one pathway may be linked to protein tyrosine kinase activation and leads to the induction of *c-fos*, *c-jun*, and other related genes, whereas a second pathway stimulates *c-myc* gene induction by an as yet unknown mechanism. We show that the latter signal is not elicited by EGFR stimulation, and growth of BAF-B03 cells bearing the EGFR cannot be supported by EGF alone. However, BAF-B03 cells bearing the EGFR can be "rescued" and made EGF responsive by ectopically expressing the human *c-myc* gene. We discuss the significance of our findings in light of mechanisms for signaling by these growth factor receptors.

## Results

### Progression of the BAF-B03 Cell Cycle by IL-2 and EGF

The murine hematopoietic cell line BAF-B03 is a subclone of the IL-3-dependent cell line BA/F3, which exhibited properties of undifferentiated pro-B cells with their immunoglobulin genes in germline configuration (Palacios and Stainmetz, 1985; Collins et al., 1988; Daley and Baltimore, 1988). We and others have shown previously that this cell line can proliferate in response to other hematopoietic cytokines including IL-2, interleukin-6 (IL-6), erythropoietin, and granulocyte colony–stimulating factor, provided that the respective cytokine receptors are expressed by cDNA transfection (Hatakeyama et al., 1989; Hibi et al., 1990; Li et al., 1990; Fukunaga et al., 1991). The following BAF-B03-derived clones were subjected to cell cycle analysis. F7 and A15 cells both express high affinity IL-2R, which is composed of endogenous IL-2Rα and cDNA-directed human IL-2Rβ. The F7 clone expresses the wild-type IL-2Rβ (about 9000 high affinity IL-2R sites), and the A15 clone expresses the human IL-2Rβ mutant, which lacks the cytoplasmic "acidic region" spanning residues 312 to 383 (about 5000 high affinity IL-2R sites) (Hatakeyama et al., 1989). Both transfectants have been shown to proliferate in response to IL-2 (Hatakeyama et al., 1989). In addition, we generated an EGFR-expressing clone BER2, by transfecting the human EGFR cDNA. BER2 expresses about 100,000 EGF-binding sites, as revealed by a [$^{125}$I]EGF binding assay (data not shown). When BER2 cells were stimulated by EGF, rapid tyrosine autophosphorylation of EGFR occurred, indicating that ligand-induced activation of the intrinsic protein tyrosine kinase proceeds satisfactorily (data not shown).

When these cells were starved for 15 hr in the absence of IL-2 or IL-3, the cells arrested in the early G1 phase of the cell cycle; flow cytometric analysis of growth factor–starved cells stained with propidium iodide showed a sharp peak, corresponding to unduplicated DNA. When the F7 cells were stimulated by IL-2 or IL-3, the cells began to replicate their DNA within 6–9 hr, showed a maximum percentage of cells in S phase after 12 hr, and entered G2/M phase 15 hr later (Figure 1). By 18 hr, the majority of cells had reentered G1. It appears that all these cells have a relatively short G2/M phase period and they grow in a less synchronous manner compared with fibroblast cells such as NIH·3T3 cells (data not shown). The A15 cells, which express the IL-2Rβ mutant, respond to IL-2 for cell cycle progression with a notable delay; however, no such change was observed when the cells were stimulated by IL-3 (data not shown). In fact, the time required for one round of IL-2-induced cell cycle progression was slightly longer for A15 than F7 cells (Figure 1). In this regard, it is important to note that these cells express a mutant IL-2Rβ lacking the acidic region that directs interaction with a src family protein tyrosine kinase ($p56^{lck}$) in COS cells (Hatakeyama et al., 1991). In BAF-B03 cells lacking the $p56^{lck}$ expression, another closely related protein tyrosine kinase $p59^{fyn}$ appears to interact with IL-2Rβ; $p59^{fyn}$ is activated by IL-2 in F7 cells. Accordingly, Western blot analysis using an anti-phosphotyrosine antibody, 468 (Wang, 1985), revealed that cellular protein tyrosine phosphorylation is rapidly induced upon IL-2 stimulation of F7 cells but not A15 cells (Y. Minami, N. Kobayashi, T. Kono, and T . T., unpublished data). Thus, IL-2-induced cell proliferation still takes place in the absence of apparent protein tyrosine phosphorylation events in this cell line. On the other hand, when BER2 cells are stimulated by EGF, the cells begin to enter S phase but fail to progress to G2/M (Figure 1). These cells died thereafter without dividing (see Figure 4A). This property was commonly found in other BAF-B03 clones similarly expressing EGFR (data not shown). Thus, EGF–EGFR interaction provokes DNA synthesis, but further progression through the cell cycle cannot be induced in this cell line, as similarly reported for the parental BA/F3 cells (Collins et al., 1988) as well as for normal bone marrow cells (Rüden and Wagner, 1988) and another IL-3-dependent line IC2 (Wang et al., 1989).

### Differential Induction of Cyclin and cdc2 Family Kinase mRNAs by IL-2, IL-3, and EGF

The above results suggest the presence of intracellular signaling pathways for BAF-B03 cell proliferation that should be distinct, at least in part, between the receptors for the hematopoietic cytokines (i.e., IL-2 and IL-3 receptors) and EGFR. In view of accumulating evidence for the critical role of cyclin and cdc2 family kinase gene expression during the cell cycle (Hunter and Pines, 1991), we next addressed the question of which of these genes can be induced in this hematopoietic cell line by IL-2, IL-3, and EGF interaction with their receptors. In fact, little is known about the induction of cell cycle genes by the new family of cytokine receptors. Blotting analysis was performed with total RNA extracted from the cells stimulated by either of the growth factors, and the results are summarized in Figure 2. Most of the cyclin mRNAs are similarly induced by IL-2 and IL-3; i.e., essentially similar levels of mRNA specific to all cyclins examined can be observed in cells following stimulation by IL-2 and IL-3, and the same is true for *cdc2* and *cdk2* mRNAs. In accordance with a previous report, cyclin D1 (*CYL1*) mRNA, which is specifically expressed in macrophages (Matsushime et al., 1991), is not

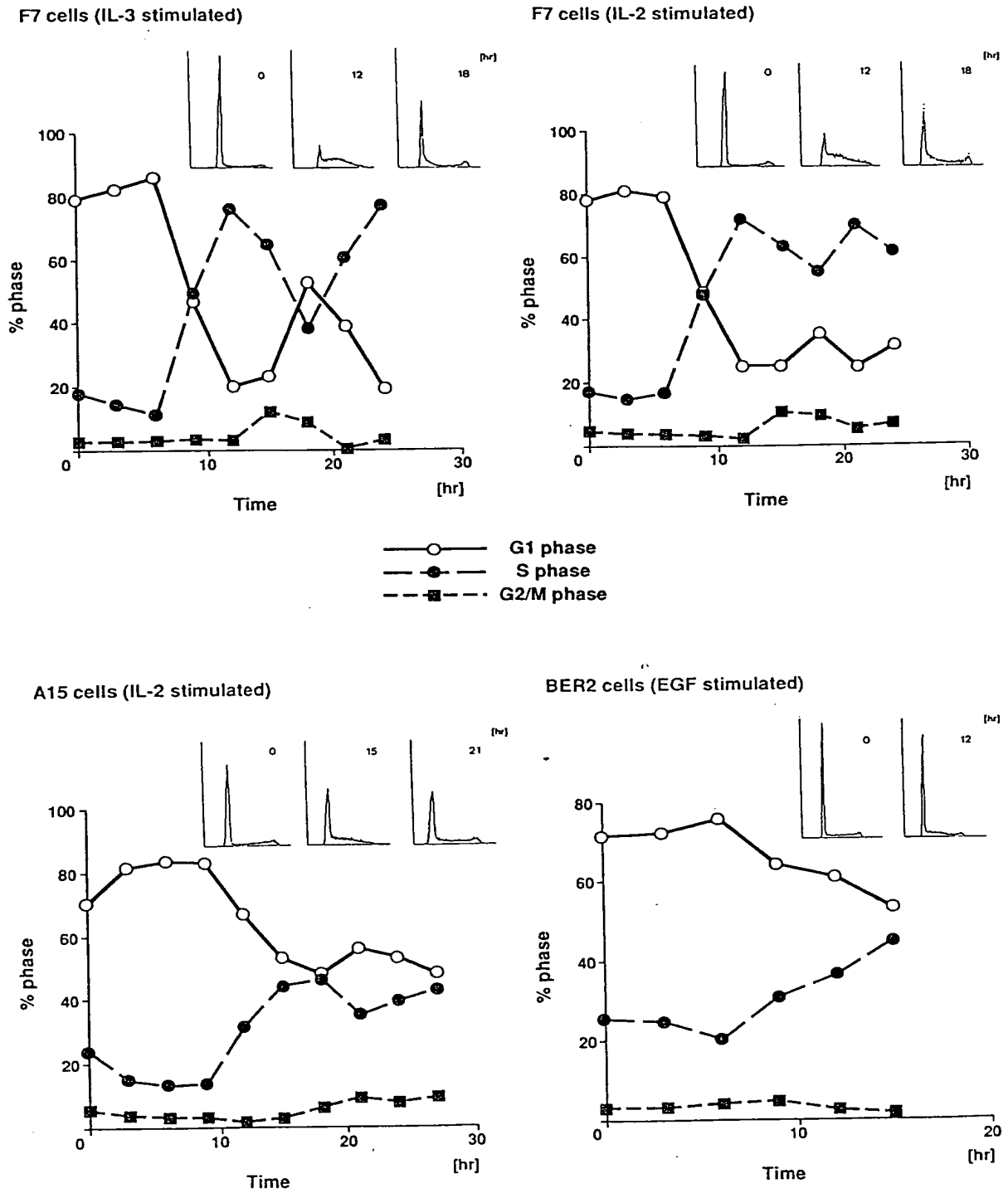
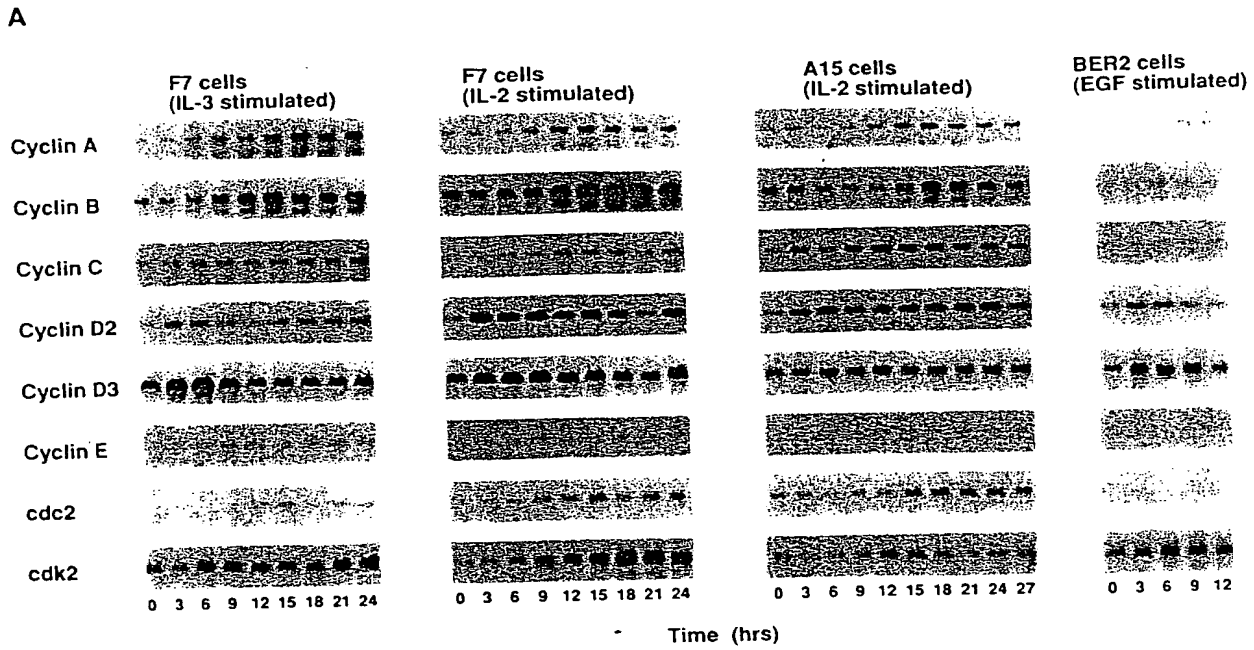



Figure 1. Cell Cycle Analysis of BAF-B03-Derived Transformant Cells

F7, A15, and BER2 cells were synchronized by growth factor starvation and stimulated with growth factors (F7 cells by IL-3 and IL-2; A15 cells by IL-2; BER2 cells by EGF). Samples were harvested at various times after stimulation, stained with propidium iodide, and analyzed by flow cytometry as described in Experimental Procedures. The calculated percentages of each phase are plotted. The histograms of flow cytometric data were indicated in the insets of each plot.



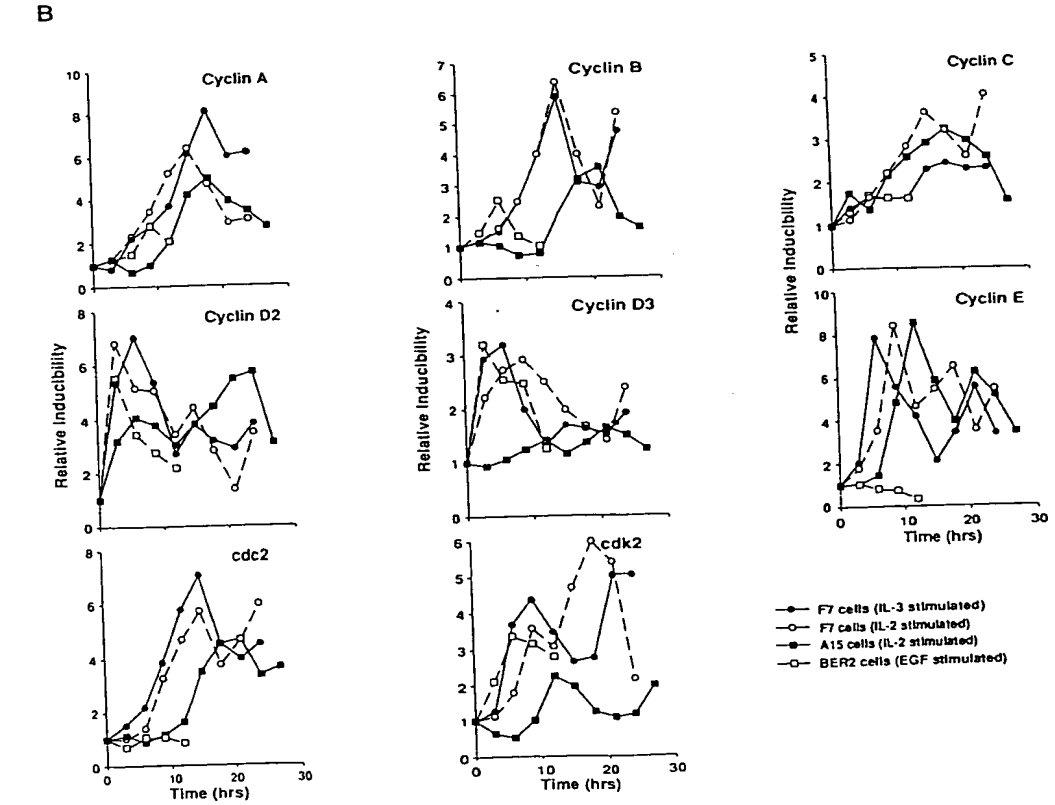




Figure 2. Differential Expression of Cyclins and cdc2 Family Kinases in BAF-B03-Derived Transformants Stimulated with IL-3, IL-2, and EGF

(A) Northern blot analysis of cyclin and cdc2 family kinase expression. Synchronized cells were stimulated with growth factors (F7 cells by IL-3 and

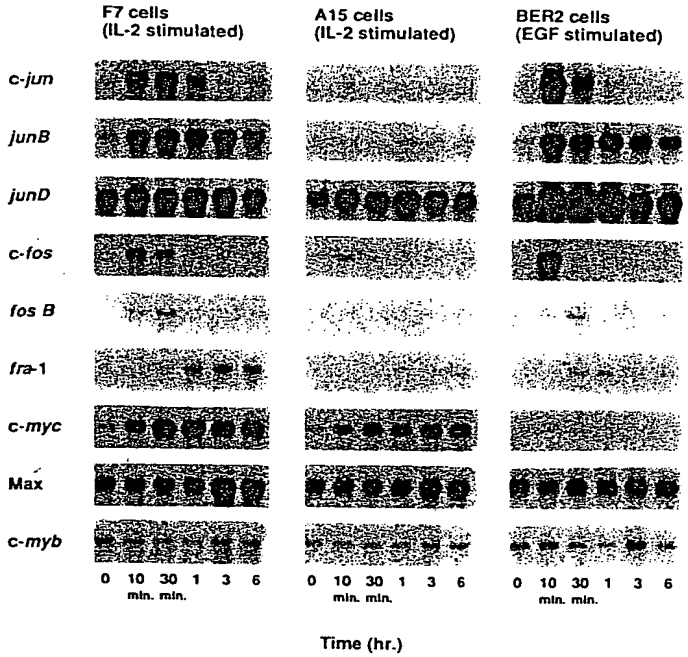



Figure 3. Differential Expression of Nuclear Proto-Oncogenes and Their Families by the Stimulation of IL-2 and EGF

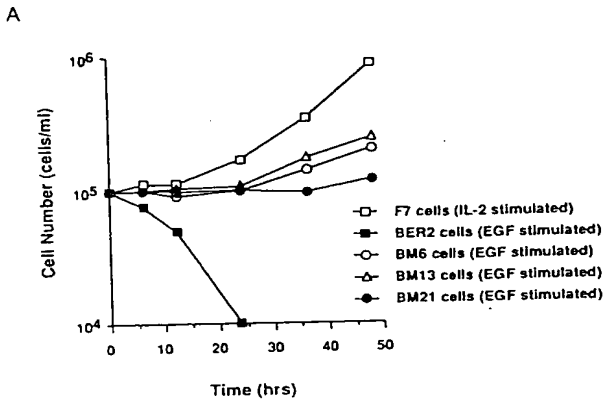
Northern blot analysis of total RNA extracted from growth factor–stimulated cells (F7 cells by IL-2; A15 cells by IL-2; BER2 cells by EGF) was performed essentially by the same procedures as for Figure 2. Membranes were exposed for 4 days (*fra-1*), 8 hr (c-*myc*), and 24 hr (others).

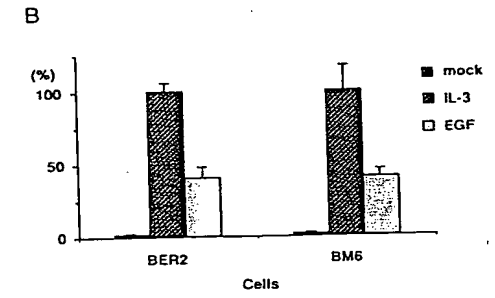
expressed in these cells (data not shown). These observations suggest that cytokine-mediated signaling through IL-2 and IL-3 receptors is similar in terms of the induction of the cyclin mRNAs. It is interesting that cyclin D3 (*CYL3*) mRNA, whose inducibility has been shown in an IL-2-dependent T lymphoid cell line but not in pre-B lymphoid and myeloid cells (Matsushime et al., 1991), is constitutively expressed and further induced by IL-2 and IL-3 in this cell line. The A15 cells bearing mutant IL-2Rβ also responded to IL-2 and expressed those mRNAs induced in IL-2-stimulated F7 cells bearing the wild-type IL-2Rβ, albeit the mRNA induction kinetics are delayed (Figure 2B). Significantly, in BER2 cells, the mRNA induction for the cyclin E and cdc2 kinase are not detectable by EGF stimulation. Furthermore, in these cells, the mRNA induction levels are notably lower for cyclins A, B, and C, as compared with the cytokine-stimulated F7 and A15 cells. Thus, among the genes known to be involved in the G1 to S transition, the EGF-induced signal(s) can be transmitted for mRNA induction of cyclins D2 and D3 and cdk2 kinase, but very poorly for cyclins C and E. On the other hand, the mRNA induction is significantly low or undetectable for the cyclins and cdc2 kinase involved in the latter phase of the cell cycle. In contrast, the latter genes are induced to the same extent as the former genes by EGF in a rat fibroblast cell line 3Y1, the proliferation of which can be induced by this growth factor (Sato et al., 1985; data not shown).

### Induction of Nuclear Proto-Oncogenes and Their Families by IL-2 and EGF

Evidence has been provided that nuclear proto-oncogenes modulate the expression of critical genes involved in cell proliferation (Hunter, 1991; Gutman and Wasylyk, 1991). One may envisage that they might influence expression of cyclin and cdc2 family kinase genes that are important in the cell cycle. In an attempt to search for a proto-oncogene(s) whose expression correlates with the induction of the mitotic cyclins and cdc2 kinase, we examined the induction of various nuclear proto-oncogenes by IL-2 and EGF. As shown in Figure 3, the expression of such genes varies intriguingly among the F7, A15, and BER2 cells. Expression of *junD*, c-*myb*, and *Max*, the partner of c-*myc*, at the RNA level is constitutive in these cells. Interestingly, c-*jun*, *junB*, c-*fos*, and *fosB* are ligand inducible in BER2 and F7 cells, but not in A15 cells expressing the IL-2Rβ chain mutant that is deficient in protein tyrosine kinase activation. In the case of c-*fos* induction by IL-2, the SRE element in the c-*fos* promoter is assumed to be the primary target for the signal (Trouche et al., 1991; Hatakeyama et al., 1992). Inductions of these genes may be linked to a tyrosine kinase–mediated pathway. In the case of *fra-1* mRNA, however, efficient induction occurs only through the wild-type IL-2Rβ in F7 cells. More intriguing is the fact that the c-*myc* gene is inducible in F7 and A15 cells, but is very poorly inducible, if at all, in BER2 cells. We have made similar observations with other BAF-B03 clones,

IL-2; A15 cells by IL-2; BER2 cells by EGF). Total RNA was extracted from them at various times. Ten micrograms from each sample was electrophoresed on a 1% agarose gel and transferred onto nylon membrane. Membranes were hybridized with radiolabeled DNA probes, washed, and exposed by autoradiography for 4 days (*cdc2*) or 24 hr (others).
(B) Autoradiogram in (A) was quantitated by densitometer scanning, and relative inducibilities were plotted in graphs.



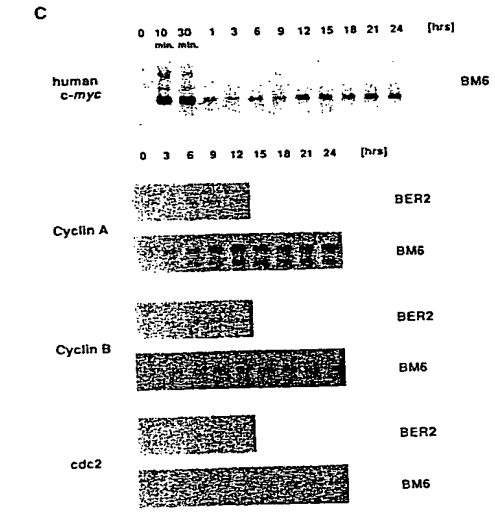




Figure 4. Expression of the Exogenous c-*myc* Gene in BER2 Cells Induces Cell Proliferation and Gene Expression of *cdc2* and Cyclins A and B

(A) Proliferation profiles for the factor-stimulated F7, BER2, BM6, BM13, and BM21 cells. Synchronized cells were plated at $1.0 \times 10^5$ cells/ml in the presence of IL-2 (F7 cells) or EGF (others). At the times shown, the density of viable cells was counted and represented on a logarithmic scale.

(B) [$^3$H]thymidine incorporation of BER2 and BM6 cells. Synchronized cells were assessed for their ability to incorporate [$^3$H]thymidine in response to IL-3 or EGF. The actual incorporation levels after IL-3 stimulation are 17,276 ± 899 cpm for BER2 cells and 20,029 ± 3,604 cpm for BM6 cells. The data are the average of triplicate determinations.

(C) Expression of the transfected human c-*myc* gene and endogenous *cdc2*, cyclin A, and cyclin B genes. Total RNA extracted from EGF-stimulated BER2 and BM6 cells was subjected to Northern blot analysis essentially by the same procedures as for Figure 2. The RNA from EGF-stimulated BER2 cells was not analyzed after 12 hr, owing to the significant loss of viable cells beyond that point.

each expressing either the wild-type or mutant IL-2Rβ or EGFR (data not shown). Therefore, the different patterns of gene induction described above are caused by the expressed receptors and are not due to clonal variation. Collectively, each of the receptors analyzed here seems to deliver signals that are partly common, but that are also distinct in terms of the induction of particular genes. The only gene among them whose inducibility correlates with the ability of the cells to enter mitosis is c-*myc*. Essentially the same pattern for the IL-2-stimulated F7 cells has been obtained in IL-3-stimulated parental BAF-B03 and F7 cells (data not shown).

### Expression of the c-*myc* Gene Renders BER2 Cells Responsive to EGF for Cell Cycle Progression

As described above, the ability of the hematopoietic BAF-B03 cells to undergo mitosis correlates with c-*myc* gene induction. While the role of the c-*myc* gene in cell growth control has been evaluated extensively (Lüscher and Eisenman, 1990), the issue of which phase of the cell cycle is most critically influenced by c-*myc* has been difficult to address, largely due to the absence of suitable experimental conditions. Our observation with the BER2 cells provided a unique opportunity to examine whether the cells, after reaching the S phase of cell cycle by EGF, can progress through the G2/M phase for mitosis by ectopically expressing the c-*myc* gene. The human c-*myc* gene was linked to the Rous sarcoma virus long terminal repeat (LTR) sequence and this chimeric gene, or a control vector, was cotransfected with the hygromycin (*hgr*) resistance gene into BER2 cells, and several *hgr*$^+$ clones were obtained. In no case were factor-independent clones obtained; the *hgr*$^+$ clones retained an IL-3 requirement for their proliferation. Interestingly, the c-*myc*-transfected clones, BM6, BM13, and BM21, also became responsive to EGF for cell proliferation in the absence of IL-3 (Figure 4A). These cells continued to proliferate in response to EGF for at least 1 week (data not shown). Supernatant from EGF-stimulated BM6 cells did not support the proliferation of BER2 or F7 cells, indicating that the EGF signal coupled with c-*myc* expression did not induce endogenous growth factors such as IL-3 whose expression is usually restricted to T lymphocytes (Arai et al., 1990). Accordingly, RNA blotting analysis with IL-3 cDNA probe did not show any IL-3 mRNA expression in BER2, BM6, BM13, and BM21 cells. Furthermore, those clones did not differ in terms of the EGFR expression level on their cell surface (data not shown). On the other hand, all the *hgr*$^+$ clones cotransfected with the control vector failed to proliferate in

response to EGF (data not shown). As shown in Figure 4B, the EGF-induced uptake levels of [$^3$H]thymidine were almost the same between the parental BER2 cells and clone BM6, suggesting that the c-*myc* gene expression in BM6 cells did not alter the levels of DNA synthesis. The EGFR is known to bind another ligand, transforming growth factor α (TGFα) (Marquardt et al., 1984). We also examined the proliferative properties of BER2 and BM6 cells induced by TGFα, and obtained essentially the same results as for EGF; TGFα induced DNA synthesis of both cells even more efficiently than EGF, but only BM6 cells progressed through the cell cycle (data not shown).

The expression of the human c-*myc* gene and other genes tested above for the parental BER2 cells was examined for the clone BM6 by RNA blotting (Figure 4C). When a probe was used that selectively hybridizes to the human c-*myc* mRNA (Dmitrovsky et al., 1986), a diagnostic band migrating at about 2.2 kb is detectable in the RNA from clone BM6. The expression level is very low during EGF starvation, but it was sharply increased 10 min after EGF stimulation, and 1 hr later the mRNA expression level went down several-fold but remained constant thereafter. It is not clear at present how the c-*myc* mRNA levels vary during the cell cycle. Presumably, this reflects the growth state–dependent control of the c-*myc* mRNA stability (Dean et al., 1986) or the promoter activity of Rous sarcoma virus LTR, or both. In this regard, it may be worth noting that the activity of another constitutive promoter, the β-actin promoter, is greatly reduced in growth-arrested BAF-B03 cells (H. S., unpublished data) and that evidence has been provided suggesting that other viral LTRs or enhancer elements are affected during the cell cycle (Imbra and Karin, 1986; Nabel and Baltimore, 1987; Doi et al., 1989b). Flow cytometric analysis of the DNA content in the EGF-stimulated BM6 cells indicated that the majority of those cells entered the G2/M phase about 15 hr after growth factor stimulation (data not shown). Similar c-*myc* gene induction was also confirmed in clones BM13 and BM21 (data not shown). Thus, the deficiency of the BER2 cells for the EGF-induced mitotic response can be overcome or rescued by the ectopic expression of the human c-*myc* gene. Concomitantly, expression of the mRNAs for cyclins A and B and cdc2 kinase genes whose expression appears to be critical for mitosis (Nurse, 1990) became up-regulated upon EGF stimulation of BM6 cells (Figure 4C). Similarly, cyclin C and E mRNAs also became inducible by EGF (data not shown).

## Discussion

Growth and differentiation of hematopoietic cells are controlled by numerous cytokines, yet the mechanism of cytokine-induced signal transduction has not been clearly elucidated. Except for the CSF-1 receptor, which exhibits protein tyrosine kinase activity, the receptors for other hematopoietic cytokines constitute a new family that functions via unknown mechanisms (D'Andrea et al., 1989; Bazan, 1990; Cosman et al., 1990; Hatakeyama, 1992). The results presented in this paper provide insights into cytokine-induced signaling pathways, as well as the nature and importance of the target genes induced by cytokines during the hematopoietic cell cycle.

### Two Distinct Signaling Pathways by IL-2Rβ

Evidence has been provided previously for the potential role of the src family kinase, p56$^{lck}$, in IL-2 signal transduction; the cytoplasmic acidic region (aa 312 to 383) of the IL-2Rβ interacts with a sequence(s) within the conserved kinase domain of the p56$^{lck}$, and IL-2 induces p56$^{lck}$ activity (Hatakeyama et al., 1991; Horak et al., 1991). In BAF-B03-derived F7 cells, another src family kinase, p59$^{fyn}$, interacts with IL-2Rβ, and this interaction presumably accounts for the IL-2-induced tyrosine phosphorylation events (Y. Minami, N. Kobayashi, T. Kono, and T. T., unpublished data).

Our results provide evidence for the presence of at least two distinct signaling pathways for the IL-2Rβ, as revealed in analyzing the target genes, i.e., nuclear proto-oncogenes as well as cyclin and the cdc2-like family of kinase genes. The tyrosine kinase pathway appears to be linked to the induction of c-*jun*, *junB*, c-*fos*, *fosB*, and *fra-1* genes, whereas the kinase-independent pathway is linked to c-*myc* gene induction. We infer that the situation will be similar or identical in normal T lymphocytes stimulated by IL-2, in view of the reports that these genes are induced by IL-2 upon proliferation (Granelli-Piperno et al., 1986; Reed et al., 1987). It is interesting to note that EGFR with intrinsic tyrosine kinase activity can efficiently induce the former set of genes such as c-*jun* and c-*fos*, but not the c-*myc* gene. These observations suggest that the tyrosine kinases of the src family and the receptor family members may share common critical substrates for signaling, or that the downstream pathways linked to the kinases later converge on common target genes. Tyrosine kinase activation in BAF-B03 cells has been reported for other cytokines (Murakami et al., 1991), but the nature of the responsible kinase is in no case clear. In view of the above argument, such kinases may not have to be src family members to evoke similar responses. On the other hand, it is interesting to note that the platelet-derived growth factor receptor has been shown to interact with src family kinases (Kypta et al., 1990). Similarly, if the EGFR could do so as well, this may provide a direct link between the IL-2R and EGFR stimulation pathways in BAF-B03 cells. Our previous data showed that a region, which is termed "serine-rich" region and is more proximal to the plasma membrane, is crucial for the IL-2Rβ to induce the proliferative response of BAF-B03 cells (Hatakeyama et al., 1989). Hence, this region may elicit an as yet unidentified signal that leads to c-*myc* gene induction. Evidence has been provided for the cooperation of c-*src* and c-*myc* genes in cellular transformation (Cleveland et al., 1989). Furthermore, it has been recently reported that, in NIH 3T3 cells, the CSF-1 receptor with a Tyr to Phe mutation at 809 is active in ligand-induced tyrosine kinase activation and in c-*fos* and *junB* gene induction, but fails to induce the c-*myc* gene (Roussel et al., 1991; see below). Hence, our present observations further suggest the bifurcation of the two growth-promoting pathways as a general feature of cell proliferation.

It is interesting that differential induction of proto-

oncogenes is reflected in the differential induction of the cell cycle genes. Induction of the c-*myc* gene correlates with the efficient induction of certain cell cycle genes such as cyclins A and B, and cdc2 kinase, believed to be critical for cells to enter G2/M phase, and their inducibility was restored by ectopic c-*myc* gene expression (Figure 4C). These results may suggest that c-*myc* functions upstream of these genes, thereby regulating them either directly or indirectly. Clearly, further work is required to elucidate the exact cascade of these gene inductions. On the other hand, the tyrosine kinase pathway seems to induce many of the cell cycle genes required to enter S phase. It may be interesting to analyze whether or not these genes are influenced by proto-oncogenes such as c-*jun* and c-*fos*.

Our results further confirm our previous notion that the coupling of the IL-2Rβ with a src family kinase is not essential for the proliferation of BAF-B03 cells (Hatakeyama et al., 1989, 1991), but at the same time they suggest the functional role of such a coupling for the full-scale activation of the proliferative machinery of the cells. In fact, the IL-2Rβ mutant lacking the acidic region is deficient in inducing tyrosine phosphorylation upon IL-2 binding, and this deficiency seems to be reflected in a delay of the induction kinetics of many cell cycle genes, as shown in Figure 2, and furthermore reflected by the slow rate of growth. This difference cannot be attributed to the slightly lower expression level of the mutant IL-2Rβ in A15 cells as mentioned above, in view of the fact that another clone F4, which expresses the wild-type IL-2Rβ much less than F7 cells (1350 high affinity sites), grows as fast as F7 cells (Hatakeyama et al., 1989). Since the mutant receptor cannot induce some of the nuclear proto-oncogenes (Figure 3), it is possible that these genes also play a role, albeit their induction is not required for this cell line to proliferate.

### EGFR and Hematopoietic Cell Proliferation

Previously, the EGFR gene was also introduced into normal bone marrow cells and other IL-3-dependent hematopoietic cell lines. In the case of the bone marrow cells and IC2 cells (Rüden and Wagner, 1988; Wang et al., 1989), the results are similar to the findings with BA/F3 (Collins et al., 1988) and BAF-B03 cells in that EGF induced DNA synthesis, but not extensive cell proliferation. In this sense, the BAF cells we have tested here manifest properties similar to normal bone marrow cells. On the other hand, in two other IL-3-dependent cell lines, 32D and FDC-P1, EGF–EGFR interaction led to cell proliferation (Pierce et al., 1988; Rüden and Wagner, 1988). Since our BER2 cells express EGFR at levels not significantly lower than those in the above cells, we think it unlikely that the receptor number could account for this difference. In this regard, it is interesting to note that one of the cell lines, FDC-P1, expresses the c-*myc* gene constitutively even after IL-3 starvation for 8 hr (Dean et al., 1987). Thus, one possibility is that in the FDC-P1 cell line, EGFR can induce the same signal(s) as in BAF-B03 cells but it can synergize or cooperate with a constitutively expressed c-*myc* gene. Alternatively, the c-*myc* target gene(s) critical for cell cycle progression may be activated in those cells.

### Induction Mechanism of c-*myc*

What is the nature of the signal that activates the c-*myc* gene? In the case of IL-2Rβ, it appears that the signal is coupled with the serine-rich region of receptor, since a deletion or point mutation in this region totally abolishes c-*myc* induction and cell proliferation (Hatakeyama et al., 1989; Mori et al., 1991; our unpublished data). In this regard, it is worth noting that in the case of the CSF-1 receptor, which has a mutation in Tyr-809 to Phe, proliferation of NIH 3T3 cells is not induced, owing to the absence of c-*myc* gene expression (Roussel et al., 1991). Since the mutant receptor is fully functional in terms of CSF-1-induced tyrosine autophosphorylation, Tyr-809 may be the recruitment site in its phosphorylated form for an important coupling molecule that transmits the signal for c-*myc* gene induction further. On the other hand, c-*myc* gene induction occurs in A15 cells in the absence of tyrosine kinase activation, suggesting that there may be at least two different pathways that are involved in the activation of this gene. In this regard, the Tyr-809 of the CSF-1 receptor and the serine-rich region of IL-2Rβ may be functioning in an analogous manner. The serine-rich region has no tyrosine residues, but an intriguing possibility exists that serine residues may go through phosphorylation to recruit a signaling molecule for c-*myc* gene induction, in analogy with the BCR gene product (Pendergast et al., 1991). Obviously, this issue requires further clarification.

### A Novel Role of c-*myc* in the Cell Cycle

Our results suggest a novel role of c-*myc* in S to G2/M phase transition and subsequent mitosis. To date, the role of nuclear proto-oncogenes during the cell cycle has not been critically assessed in mammalian cells, owing to the lack of suitable experimental systems. Our results of human c-*myc* gene expression in BER2 cells clearly show that neither EGF signaling nor c-*myc* gene expression is sufficient for the mitotic response of the cells. Thus, it is obvious that the function of the IL-2Rβ in cell proliferation is not restricted to the induction of the c-*myc* gene.

In fact, a virus LTR-driven, human c-*myc* gene expression caused the EGF-stimulated BM6 cells to progress the cell cycle further after S phase, without affecting the DNA synthesis level induced by EGF, as revealed in the [$^3$H]thymidine uptake assay (Figure 4B). In fact, the c-*myc* gene expression induced by IL-2 in F7 and A15 cells occurs rapidly, reaches a peak in 1 hr (F7 cells) or 3 hr (A15 cells), but thereafter certain levels of the mRNA expression persist for the rest of the cell cycle (Figure 3; data not shown). This is true for other cells and in fact suggests the potential role of c-*myc* in later phases of the cell cycle, presumably in collaboration with *Max*, whose expression is usually constitutive (Blackwood et al., 1992). What is the role of c-*myc* in the S to G2/M transition? Presumably, it affects more than one of the genes that are critical for the progression of the cell cycle at this phase; however, it is not clear exactly when in the cell cycle c-*myc* expression is required. An attempt to rescue the BER2 cells for the EGF response by ectopically expressing the cdc2 kinase cDNA has not been successful (H. S., unpublished data).

Hence, further work is required to assess the functional mechanism of c-*myc* in the S to G2/M transition. Our results by no means exclude the possibility that c-*myc* also plays a role in the G1 to S transition. In fact, evidence suggests that c-*myc* may assist in regulating DNA synthesis (Lüscher and Eisenman, 1990; Hunter, 1991). It is possible that a very low level of c-*myc* induction soon after EGF stimulation of BER2 cells is sufficient for the cells to enter S phase but not for subsequent cell cycle progression, in view of the unstable nature of the protein product (Blackwood et al., 1992). Alternatively, it is also possible that the target gene(s) of c-*myc* critical for DNA synthesis is activated in BAF-B03 cells.

In conclusion, our experimental approach may have opened a way to clarify the nature of signaling pathways by hematopoietic cytokines and EGF, which involve the induction of nuclear proto-oncogenes and their families as well as cyclin and cdc2 family kinase genes, and point to the hitherto uncovered role for c-*myc* in the cell cycle.

## Experimental Procedures

### Cell and Cell Culture

BAF-B03, a subclone of BA/F3, is a bone marrow–derived murine IL-3-dependent pro-B cell line (Palacios and Steinmetz, 1985). It exhibits the following profile of cell surface marker expression: Mac-1$^+$, Bp1$^-$, B220$^-$, sIgM$^-$, Lyb1$^-$, Thy1$^-$, Ly1$^-$, L3T4(CD4)$^-$, Lyt2(CD8)$^-$, and IL-2Rα$^+$ (Hatakeyama et al., 1989). F7 and A15 are BAF-B03-derived stable transfectant clones expressing wild-type human IL-2Rβ and the IL-2Rβ mutant lacking the internal acidic region, respectively (Hatakeyama et al., 1989). BER2 is also a BAF-B03-derived clone that was obtained by transfecting the plasmid in which human EGFR cDNA was inserted into pNeoSRαII (kindly provided by Dr. Atsushi Miyajima, DNAX Research Institute, Palo Alto, CA; Wang et al., 1989). Clones BM6, 13, and 21 were obtained by transfecting the human c-*myc* expression plasmid, pN-LTRmyc (containing the second and third exons of the genomic human c-*myc* gene; Battey et al., 1983), into BER2 cells. Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 10% (v/v) conditioned medium from the WEHI-3B cell line (10% WEHI conditioned medium) as a source of IL-3.

Cells were synchronized in G1 phase by depriving them of cytokines for 15 hr. To analyze cell cycle progression and gene expression, cells were stimulated with IL-3 (10% WEHI conditioned medium) for F7 cells, recombinant human IL-2 (2 nM) for F7 and A15 cells, and recombinant human EGF (10 ng/ml) for BER2 and BM cells.

### DNA Transfection

Plasmid DNAs were transfected into the cells by an electroporation procedure as described previously (Doi et al., 1989a). Selection was initiated 24 hr after the transfection, using 2 mg/ml G418 for BER2 cells or 1 mg/ml hygromycin for BM cells in 10% WEHI conditioned medium. Cells were cultured in 24-well microculture plates. Drug-resistant colonies were picked up and subsequently cloned by limiting dilution as described previously (Hatakeyama et al., 1989).

### Cell Cycle Analysis

Essentially, the analysis was done by following the protocol provided by CycleTEST, Becton Dickinson (Vindeløv et al., 1983). In brief, cells were suspended in citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO) ($2.5 \times 10^6$ cells/ml) and treated with trypsin to solubilize cell membranes. After mixing for 10 min at room temperature, the solution containing trypsin inhibitor and ribonuclease A was added. The solutions were again mixed at room temperature, and after 10 min, ice-cold solution containing propidium iodide was added to stain the nuclei (final propidium iodide concentration, 125 μg/ml). Fluorescence intensity in each cell nucleus was measured with a FACScan (Becton Dickinson). Approximately 20,000 cells were analyzed from each sample at a rate of 50 to 100 cells per second. The percentages of cells in each phase of the cell cycle were determined by analysis with the computer program CellFIT (Becton Dickinson).

### Preparation of Probe DNA

The probe DNAs for *cdc2*, *cdk2*, c-*jun*, *junB*, *junD*, c-*fos*, *fra-1*, c-*myc*, and c-*myb* mRNAs were prepared as follows: *cdc2*, 1.6 kb EcoRI fragment excised from pcdc2, which contains human *cdc2* cDNA (Ninomiya-Tsuji et al., 1991); *cdk2*, 1.5 kb EcoRI fragment from the plasmid in which human *cdk2* cDNA is inserted into pBluescript II SK(+) (Ninomiya-Tsuji et al., 1991); c-*jun*, 1.0 kb FspI–HincII fragment from pSP-c-jun (Rauscher et al., 1988); *junB*, 0.5 kb EcoT14I–SacI fragment from the plasmid that contains mouse *junB* cDNA in pBluescript KS(+) (Hirai et al., 1989); *junD*, 0.9 kb SphI fragment from the plasmid that contains mouse *junD* cDNA in pBluescript(+) (Hirai et al., 1989); c-*fos*, 0.4 kb StuI–SacI fragment from pSV-mFOS (Hatakeyama et al., 1992); *fra-1*, 260 bp KpnI–BstXI fragment from pSP-fra-1 (Cohen and Curran, 1988); c-*myc*, 1.9 kb HindIII fragment from pMc-myc54 (Stanton et al., 1983); c-*myb*, 2.0 kb EcoRI fragment of the human c-*myb* gene from pcmybE was inserted into pBR322 (Franchini et al., 1983). The plasmids containing c-*jun*, *junB*, *junD*, and *fra-1* cDNAs were kindly provided by Dr. Iba (Institute of Medical Science, University of Tokyo, Japan). Cyclin A, B, C, D2, D3, E and *Max* and *fosB* cDNAs were obtained by the polymerase chain reaction cloning method using the synthesized primers from the published cDNA sequences (cyclin A, Wang et al., 1990; cyclin B, Pines and Hunter, 1989; cyclin C and E, Lew et al., 1991; cyclin D2 and D3, Matsushime et al., 1991; *Max*, Prendergast et al., 1991; *fosB*, Zerial et al., 1989), and the cDNAs were each subcloned into the HincII site of the pUC19 vector. cDNA fragments excised from these plasmids were used as probes. To detect the expression of human c-*myc*, a 1.5 kb ClaI–EcoRI fragment from the third exon of pN-LTRmyc was used as a human c-*myc*-specific probe (Dmitrovsky et al., 1986).

### RNA Extraction and Northern Blot Analysis

Total cellular RNA from each cell was prepared by denaturation in guanidium thiocyanate followed by pelleting through a cesium chloride cushion (Shibuya et al., 1990). For Northern blot analysis, 10 μg of total RNA was electrophoresed on 1% agarose formaldehyde gels and transferred onto nylon membranes. Probes were labeled with [α-$^{32}$P]dCTP using a Multiprime labeling kit (Amersham) and hybridized as described previously (Harada et al., 1990). Specific activity was approximately $1 \times 10^8$ cpm/ng for all the probe DNAs.

### Measurement of the Cell Number Increase

Synchronized cells were cultured at a density of $1 \times 10^5$/ml (5 ml of culture) in RPMI 1640 supplemented with 10% fetal calf serum containing recombinant human IL-2 (2 nM) (F7 cells) or recombinant human EGF (10 ng/ml) (BER2 and BM cells). Viable cell counts were determined by trypan blue staining.

### Measurement of [$^3$H]Thymidine Incorporation

Cells ($2 \times 10^4$) were cultured without growth factors in RPMI 1640/10% fetal calf serum in a 96-well microculture plate for 15 hr. After stimulation with recombinant human IL-2 (2 nM) or recombinant human EGF (10 ng/ml), cells were pulsed with 1 μCi of [$^3$H]thymidine for 4 hr prior to harvest. [$^3$H]thymidine incorporation was measured as described previously (Hatakeyama et al., 1989).

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# Three Distinct IL-2 Signaling Pathways Mediated by *bcl-2*, *c-myc*, and *lck* Cooperate in Hematopoietic Cell Proliferation

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## Summary

**Two interleukin-2 receptor–dependent signaling pathways have thus far been identified: the c-*fos*/c-*jun* induction pathway mediated by *src* family protein-tyrosine kinases and the c-*myc* induction pathway. Here, we provide evidence for the existence of a third, rapamycin-sensitive pathway, which results in the induction of another proto-oncogene, *bcl-2*. In the hematopoietic cell line BAF-B03, the expression of any two of *lck*F505 (an active form of p56$^{lck}$), Bcl-2, or c-Myc is sufficient to promote transit of the cell cycle, regardless of the activation state of the third pathway. We also provide evidence that epidermal growth factor receptor signaling may act through the same pathway that involves p56$^{lck}$. These studies demonstrate a novel approach to dissecting signaling pathways regulating cellular proliferation.**

## Introduction

The functional interleukin-2 receptor (IL-2R) consists of three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγ chains, none of which contain an intrinsic protein-tyrosine kinase (PTK) domain that is the hallmark of other growth factor receptors (reviewed by Waldmann, 1989; Ullrich and Schlessinger, 1990; Taniguchi and Minami, 1993). We have shown previously that expression of the human IL-2Rβ cDNA in BAF-B03, an interleukin-3 (IL-3)-dependent murine hematopoietic cell line that expresses IL-2Rα and IL-2Rγ but not IL-2Rβ, allows the cells to proliferate in response to IL-2. Expression studies with mutant human IL-2Rβ cDNAs have revealed that a mutant (S mutant) lacking the membrane-proximal cytoplasmic region, termed the serine-rich region (S region), cannot transmit proliferative signals (Hatakeyama et al., 1989). More recently, evidence has also been provided demonstrating the critical role of the IL-2Rγ cytoplasmic region in IL-2 signaling (Kawahara et al., 1994; Nakamura et al., 1994; Nelson et al., 1994), pointing to the importance of cooperation between the IL-2Rβ and IL-2Rγ cytoplasmic domains in activating downstream signaling pathways. Despite extensive structural and functional studies on the IL-2R components, information is still limited with respect to the nature and function of the signaling pathways that they activate and their target genes.

Although the IL-2R components themselves lack PTK domains, IL-2 stimulation evokes tyrosine phosphorylation of intracellular proteins. Notably, the IL-2Rβ chain interacts both physically and functionally with members of the *src* family of nonreceptor PTKs; p56$^{lck}$ is activated by IL-2 in peripheral blood lymphocytes (PBLs) (Hatakeyama et al., 1991; Horak et al., 1991), and other *src* family members, p59$^{fyn}$ and p53/56$^{lyn}$, are activated in an analogous manner in BAF-B03-derived cells (Torigoe et al., 1992; Kobayashi et al., 1993). The physical interaction between p56$^{lck}$ and IL-2Rβ is mediated by the PTK domain and the "acidic region" (A region), respectively (Hatakeyama et al., 1991). Moreover, the activation of *src* family PTKs by IL-2 requires the S region of IL-2Rβ, and this activation correlates both with p21$^{ras}$ activation (Satoh et al., 1992) and with induction of the nuclear proto-oncogenes c-*fos* and c-*jun* (Shibuya et al., 1992; Minami et al., 1993). Recently, it was found that the S region of IL-2Rβ is necessary for the binding of two distinct PTKs, Jak1 of the Janus family PTKs (Miyazaki et al., 1994; Witthuhn et al., 1994) and Syk PTK of the Syk/ZAP-70 family PTKs (Minami et al., 1995) and that the carboxy-terminal region of IL-2Rγ is necessary for the association of Jak3 (Johnston et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). However, the role of these PTKs in the activation of *src* family PTKs is still unknown. A mutant form of the IL-2Rβ chain lacking the A region (A mutant) is deficient in activating the p56$^{lck}$ PTK pathway (Minami et al., 1993), but it is still capable of bringing about IL-2-induced proliferation when expressed in BAF-B03 cells (Hatakeyama et al., 1989). Hence, this raises the issue of whether the p56$^{lck}$ PTK pathway is involved in any way in IL-2-induced cell proliferation.

Expression studies with mutant IL-2Rβ and epidermal growth factor receptor (EGFR) cDNAs in BAF-B03 cells revealed the presence of another IL-2R-linked pathway

leading to the induction of the nuclear proto-oncogene c-*myc* (Shibuya et al., 1992). This pathway is activated by the wild-type and the A mutant but not by the S mutant IL-2Rβ chains. EGFR stimulation results in the activation of the c-*fos*/c-*jun* pathway but not the c-*myc* pathway, and the c-*myc* gene must be ectopically expressed for the EGF-stimulated cells to progress beyond S phase of the cell cycle (Shibuya et al., 1992). Hence, c-*myc* is a target of the IL-2R-mediated signal that is required for the progression of the hematopoietic cell cycle. Constitutive expression of c-*myc*, however, does not cause IL-2 (or IL-3)-independent growth of BAF-B03 cells (Shibuya et al., 1992). Taken together, these observations indicate that an additional, as yet unidentified target gene(s) is required for cell proliferation. This gene is activated by the IL-2R, does not respond to p56$^{lck}$, and must cooperate with c-*myc* (see Figure 1).

In the present study, we have reexamined the genes involved in BAF-B03 cell proliferation and have adduced evidence that *bcl-2* is another target gene that mediates IL-2-induced cell proliferation. *bcl-2* was originally identified as an oncogene that is amplified in follicular lymphoma (Tsujimoto and Croce, 1986), and it has been extensively studied in the context of the inhibition of apoptosis (reviewed by Korsmeyer, 1992). Recently, in addition to *bcl-2*, a *bcl-2*-related gene, *bcl-x*$_L$, has also been demonstrated to inhibit apoptosis (Boise et al., 1993). In contrast, another *bcl-2*-related gene, *bax*, has been shown to accelerate apoptosis (Oltvai et al., 1993). The induction of *bcl-2*, *bcl-x*$_L$, or *bax* by IL-2 also requires the S region of the IL-2Rβ chain; however, only *bcl-2* induction is inhibited by rapamycin (RAP) (Figure 1). We demonstrate that c-*myc* and *bcl-2* can cooperate in stimulating cell proliferation, in that coexpression of these two genes leads IL-2 (IL-3)-independent cell proliferation. Moreover, we observed that an activated form of p56$^{lck}$ carrying a phenylalanine substitution at position 505 (p56$^{lck}$F505) can cooperate with either c-Myc or Bcl-2 in the induction of cell proliferation, providing strong evidence supporting a role for p56$^{lck}$ in transmitting mitogenic signals. We also examined cooperation of either of p56$^{lck}$F505, c-Myc, or Bcl-2 with EGFR stimulation.

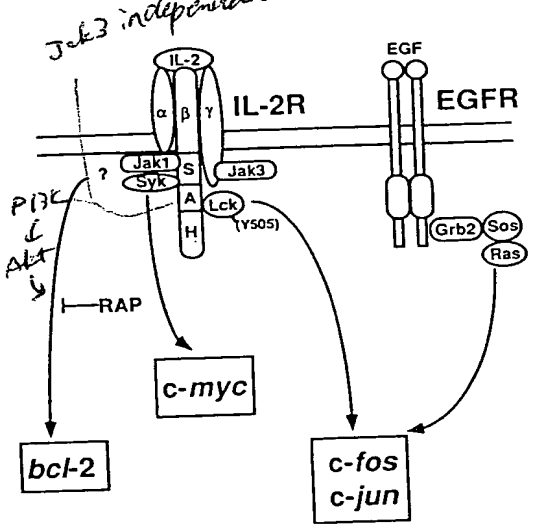


Figure 1. A Model for Three Distinct Signaling Pathways Diverging from IL-2R

This figure summarizes our conclusion that IL-2R stimulation is linked to three distinct signaling pathways: the p56$^{lck}$ activation pathway (which leads to c-*fos*/c-*jun* induction), the c-*myc* induction pathway, and the RAP-sensitive, *bcl-2* induction pathway. The physical interaction between p56$^{lck}$ and IL-2Rβ requires the A region of the IL-2Rβ chain. Induction of both the c-*myc* and *bcl-2* genes is mediated by the S region of the IL-2Rβ chain. The S region of IL-2Rβ is necessary for binding of two distinct PTKs, Jak1 of the Janus family PTKs and Syk PTK of the Syk/ZAP-70 family PTKs. The activation of Syk PTK is linked to the induction of the c-*myc* gene. The H region of IL-2Rβ is essential for Stat5 activation. The carboxy-terminal region of IL-2Rγ is necessary for the association with Jak3. The role of Jak1 and Jak3 PTKs in the induction of these proto-oncogenes remains unknown (see text for details).

## Results

### Induction of *bcl-2* Gene by IL-2R

It has been shown that many hematopoietic cell lines undergo apoptosis in the absence of cytokine stimulation and that the product of the *bcl-2* gene prolongs cell survival and blocks the apoptosis (reviewed by Korsmeyer, 1992; Collins and Rivas, 1993; Vaux, 1993). It has been demonstrated also that IL-2 stimulation induces *bcl-2* gene expression (Deng and Podack, 1993; Otani et al., 1993); however, a role for Bcl-2 in IL-2 signaling is not well established.

To examine if and how the *bcl-2* gene is involved in IL-2-induced cell proliferation, we first studied *bcl-2* mRNA induction in BAF-B03 cells expressing wild-type or mutant IL-2Rβ chains (i.e., F7, A15, and S25 cells). The F7 cells express the wild-type IL-2Rβ chain, the A15 cells express a mutant IL-2Rβ chain lacking the A region (amino acid residues 313–382), and the S25 cells express an IL-2Rβ chain lacking the S region (amino acid residues 267–322). It has been previously shown that A15 cells can respond to IL-2, albeit less well than F7 cells, whereas S25 cells remain unresponsive (Hatakeyama et al., 1989). When F7 cells were stimulated with IL-2, *bcl-2* mRNA induction could be seen after 30 min and reached its peak level after 12 hr (Figure 2). The induction of the *bcl-2* gene was also observed in A15 cells after IL-2 stimulation, albeit at a lower level than in F7 cells. IL-2 stimulation of S25 cells, however, did not result in *bcl-2* mRNA expression (Figure 2). These results indicate that the S region of IL-2Rβ, which is essential for growth signal transmission, is also necessary for *bcl-2* gene induction. The S region was also found to be essential for the induction of the *bcl-x*$_L$ and *bax* genes (Figure 2; data not shown).

### *bcl-2* Gene Induction Is Sensitive to Rapamycin

To dissect the IL-2 signaling pathways further, we next examined the effects of two immunosuppressive drugs, FK506 and RAP, on IL-2-induced proto-oncogene expression. These drugs are known to bind to the same intracellular target, but the latter selectively affects the IL-2-dependent T cell proliferation (Sigal and Dumont, 1992). As shown in Figure 3A, neither of the drugs showed any effect on the induction of c-*fos*, c-*jun*, or c-*myc* genes by IL-2.

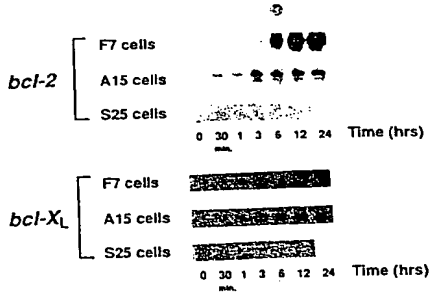


Figure 2. Expression of the *bcl-2* and *bcl-x*$_L$ Genes in BAF-B03-Derived Transformants Stimulated with IL-2

Northern blot analysis of mRNA expression in F7, A15, and S25 cells. Total RNA was extracted from cells that had been synchronized (by factor starvation for 15 hr) and then stimulated with IL-2 (2 nM) for various periods. Northern blot analysis was carried out as described in Experimental Procedures, and autoradiographs were exposed for 3 days. Expression of Bcl-2 protein was confirmed by Western blot analysis.

On the other hand, RAP but not FK506 was found to selectively retard *bcl-2* gene induction in a profound manner; when F7 cells were stimulated with IL-2 in the presence of RAP, the expression of *bcl-2* mRNA was delayed for 6–9 hr. However, RAP showed no effect on the induction of *bcl-x*$_L$ and *bax* mRNAs (Figure 3B; data not shown). This suggests that *bcl-2* gene induction may be one of the targets of RAP. IL-2-induced cell cycle progression as well as the induction of some cell cycle stage–specific genes was also affected by RAP (data not shown). There is thus a close correlation between the IL-2-induced expression of the *bcl-2* gene and progression through the cell cycle.

### *bcl-2* Cooperates with EGF Signaling to Elicit Cell Proliferation in a c-*myc*-Independent Manner

It has been shown previously that, in BAF-B03-derived BER2 cells, c-*myc* plays a critical role in the progression of the cell cycle up to the G2/M phase. BER2 cells have been engineered to express the human EGFR cDNA, and EGF stimulation causes the uptake of [$^3$H]thymidine, but does not elicit cell cycle progression, unless c-*myc* is also expressed ectopically (Shibuya et al., 1992). In view of the fact that neither the c-*myc* gene nor the *bcl-2* gene is induced by EGF stimulation in BER2 cells (Shibuya et al., 1992; Figure 4A[a]), we tested the ability of *bcl-2* to drive cell cycle progression in BER2 cells. The human *bcl-2* cDNA was cloned into the expression vector pSVT, and the resulting construct pSVBT was cotransfected with the hygromycin (*hgr*) resistance gene into BER2 cells. Three clones expressing the transfected *bcl-2* cDNA, BB3, BB8, and BB13, were obtained. These clones did not proliferate in a growth factor–independent fashion but could be induced to proliferate in response to EGF stimulation (Figure 4B). The BB cells continued to proliferate in response to EGF stimulation for at least 1 month. All the *hgr*$^+$ clones that were cotransfected with the control vector pSVT failed to proliferate in response to EGF (data not shown).

RNA blotting analysis with the mouse IL-3 cDNA probe did not show any IL-3 mRNA expression in BB3, BB8, and BB13 cells cultured in the EGF-containing medium.

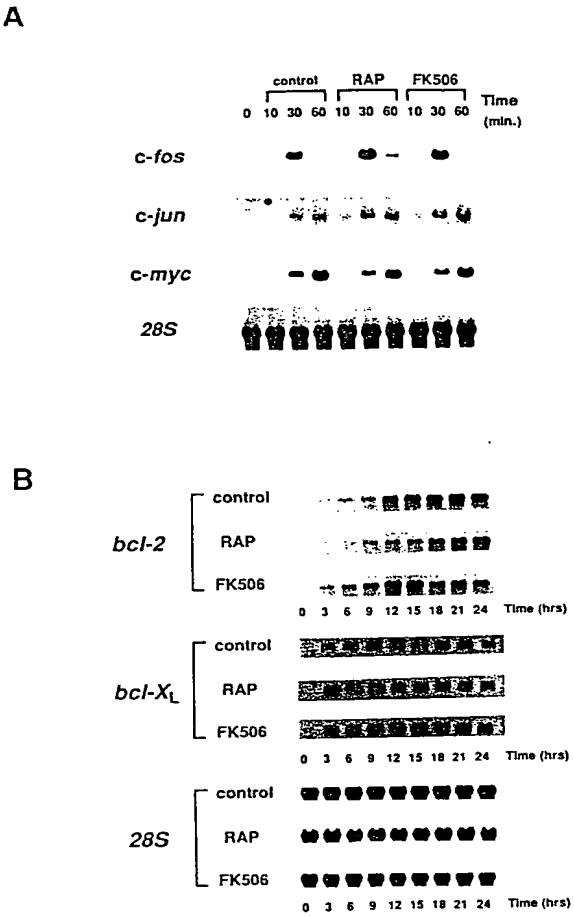


Figure 3. Effects of RAP on the Expression of Proto-Oncogenes and *bcl-x*$_L$ Gene Induced by IL-2

(A) RAP has no effect on the expression of c-*fos*, c-*jun*, and c-*myc* proto-oncogenes in F7 cells following IL-2 stimulation. Synchronized F7 cells ($2 \times 10^7$) were preincubated with culture medium or culture medium containing either RAP (10 ng/ml, Wyeth-Ayerst Laboratories) or FK506 (10 ng/ml, Fujisawa Chemical) for 30 min and stimulated with IL-2 (2 nM) for various periods. Northern blot analysis was carried out as described in Figure 2. Autoradiographic exposure times were 3 hr (c-*myc*), 3 days (c-*fos*), and 6 days (c-*jun*). Membranes were stained with methylene blue and stained 28S ribosomal RNA was indicated.
(B) RAP affects the *bcl-2* gene but not *bcl-x*$_L$ gene induction in F7 cells by IL-2 stimulation. Synchronized F7 cells ($2 \times 10^7$) were preincubated with culture medium or culture medium containing the respective drugs RAP (10 ng/ml) or FK506 (10 ng/ml) for 30 min and stimulated with IL-2 (2 nM) for various periods. Autoradiographic exposure times were 1 day (*bcl-2*) and 3 days (*bcl-x*$_L$). Membranes were stained with methylene blue and stained 28S ribosomal RNA was indicated.

In addition, supernatant from EGF-stimulated BB3, BB8, and BB13 cells did not support the proliferation of BER2 or F7 cells (data not shown), indicating that cell proliferation is directly mediated by the combination of EGF signaling and *bcl-2* expression rather than the induction of growth stimulatory cytokines. EGF stimulation of BER2 cells did not induce the N-*myc*, *bcl-x*$_L$, or *bax* genes (Figure 4A[a]; data not shown). Importantly, EGF stimulation of BB3 cells did not induce c-*myc* or N-*myc* expression (Figure 4A[b]),

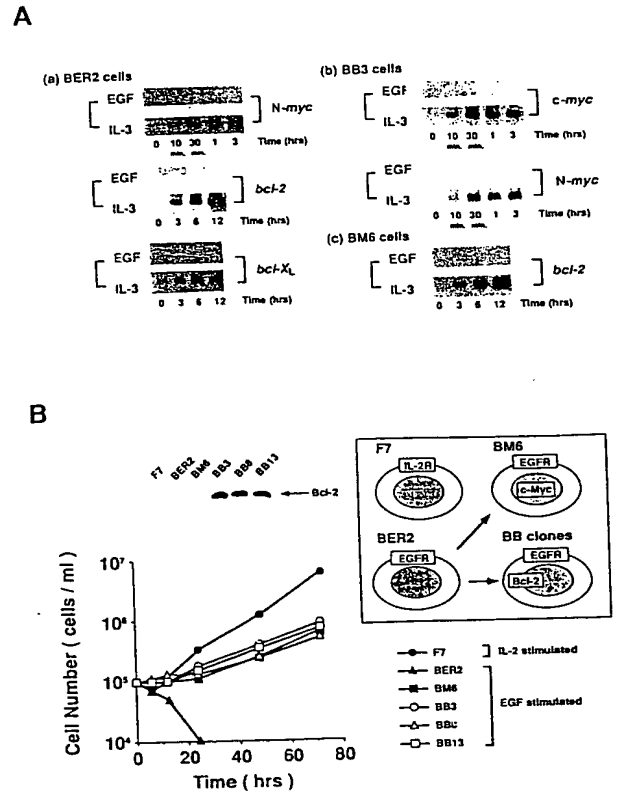


Figure 4. Expression of the Exogenous *bcl-2* Gene in BER2 cells Has No Effect on *myc* Genes Induction but Confers Responsiveness to EGF for Cell Proliferation

(A) (a) Expression of N-*myc*, *bcl-2*, and *bcl-x*$_L$ genes in factor–stimulated BER2 cells. After synchronized BER2 cells were stimulated with either EGF or IL-3 for various periods, total RNA extracted from them was subjected to Northern blot analysis.

(b) Expression of c-*myc* and N-*myc* genes in factor-stimulated BB3 cells. After synchronized BB3 cells were stimulated with either EGF or IL-3 for various periods, total RNA extracted from them was subjected to Northern blot analysis.

(c) Expression of *bcl-2* gene in factor-stimulated BM6 cells. After synchronized BM6 cells were stimulated with either EGF or IL-3 for various periods, total RNA extracted from them was subjected to Northern blot analysis.

(B) Proliferation profiles for the factor-stimulated F7, BER2, BM6, BB3, BB8, and BB13 cells. Expression of the human Bcl-2 was assessed by Western blot analysis and is indicated by the arrow (left inset). Synchronized cells were plated at 1 × $10^5$ cells/ml in the presence of IL-2 (F7 cells) or EGF (others). The number of viable cells was counted at various times after factor stimulation and represented on a logarithmic scale. The IL-3R is not shown in the right inset panel.

suggesting that cell proliferation indeed occurs in the absence of *myc* gene expression. Uptake of [$^3$H]thymidine following EGF stimulation was almost the same between the parental BER2 cells and the BB3 clone (data not shown), suggesting that the expression of the *bcl-2* gene in these clones did not alter the levels of DNA synthesis. Thus, progression of the cell cycle in BAF cells can be brought about by the cooperation between EGF signaling and either c-*myc* or *bcl-2* expression.

We also examined whether expression of the endogenous *bcl-2* gene is affected by c-*myc* expression. The BER2-derived BM6 cells constitutively express human c-Myc from a transfected expression plasmid containing the human c-*myc* gene, and these cells continued to proliferate in response to EGF stimulation for at least 1 month (Shibuya et al., 1992; data not shown). As shown in Figure 4A(c), *bcl-2* mRNA induction was not detectable in BM6 cells after EGF stimulation. These observations indicate that the c-*myc* and *bcl-2* genes do not affect the expression of each other and, therefore, that Bcl-2 and c-Myc independently cooperate with EGFR stimulation to promote the cell cycle progression.

### Cooperation of c-Myc and Bcl-2 Enables BAF-B03 Cells to Proliferate Independent of Cytokines

To clarify further the nature of the cooperation between Bcl-2 and c-Myc in cell proliferation, we generated cell transformants that constitutively express both the human c-*myc* and *bcl-2* genes (BMB). The puromycin (*puro*) resistance gene and either an expression vector for human *bcl-2* gene (pSVBT) or control vector (pSVT) were cotransfected into a cell line, BM6. Whereas the parental BM6 cells were unable to proliferate unless they were stimulated with either EGF or IL-3, the *puro*$^+$ clones BMB7, BMB17, and BMB23, which express the transfected *bcl-2* gene, could proliferate in a growth factor–independent fashion (Figure 5A). BMB cells continued to proliferate in the absence of either IL-3 or EGF for at least 1 month at a rate approximately 70% of that of the IL-3-stimulated parental BER2 cells (data not shown). Evidence for the cooperation of c-*myc* and *bcl-2* in lymphoid tumorigenesis has been provided previously (Vaux et al., 1988; Strasser et al., 1990).

### Cooperation of p56$^{lck}$ PTK with c-Myc or Bcl-2 for Cell Proliferation

To address the issue of whether the *src* family PTKs contribute to IL-2-induced cell proliferation, we next examined whether p56$^{lck}$ could cooperate with either of the two known target genes of IL-2 signaling, c-*myc* and *bcl-2*. This seemed especially important to test since the induction of c-*myc* and *bcl-2* genes occurs in A15 cells that express a mutant IL-2Rβ deficient for the *src* family PTK activation and since the activation of the *src* family PTKs, like the activation of an artificially introduced EGFR, leads to the induction of the c-*fos*/c-*jun* genes, but not the c-*myc* and *bcl-2* genes (Shibuya et al., 1992; Minami et al., 1993; Figures 2 and 4A[a]; T. M., unpublished data).

Mutation of the conserved carboxy-terminal tyrosine residue (505) of p56$^{lck}$ to phenylalanine (p56$^{lck}$F505) gives rise to a p56$^{lck}$ variant with much increased PTK activity (Marth et al., 1988). An expression vector for p56$^{lck}$F505, termed pdKCR-lckF505, was constructed and cotransfected with the *hgr* resistance gene into BAF-B03-derived BER2 cells expressing the human EGFR (Shibuya et al., 1992). Several transformants expressing the active form of p56$^{lck}$ (p56$^{lck}$F505) were obtained, and these clones were termed BF cells. These BF clones were unable to proliferate upon EGF stimulation, indicating the lack (or insufficiency) of cooperation between EGFR signaling and p56$^{lck}$

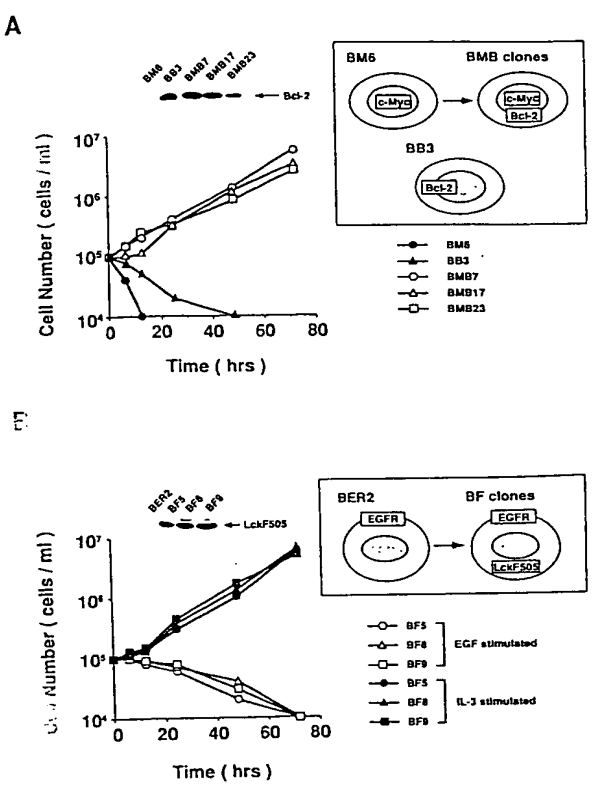


Figure 5. Proliferation Profiles for BMB and BF Cells

(A) Factor independency of the BMB7, BMB17, and BMB23 cells for cell growth. Expression of Bcl-2 was assessed by Western blot analysis and is indicated by the arrow (left inset). Synchronized BM6, BB3, and BMB cells were plated at 1 × $10^5$ cells/ml in the absence of IL-2, IL-3, and EGF. Coexpression of the c-*myc* and *bcl-2* genes in BAF-B03 cells also resulted in IL-3-independent proliferation. These cells did not express detectable levels of IL-3 mRNA by RNA blotting analysis, and the culture supernatant was unable to support proliferation of BER2 or F7 cells, thus indicating that the cell proliferation observed is not mediated by endogenously produced growth factors.

(B) Insufficiency of cooperation between EGF signaling and p56$^{lck}$F505 in BF cells. Expression of p56$^{lck}$F505 was assessed by Western blot analysis and is indicated by the arrow (left inset). Synchronized BF cells were plated at 1 × $10^5$ cells/ml in the presence of IL-3 or EGF.

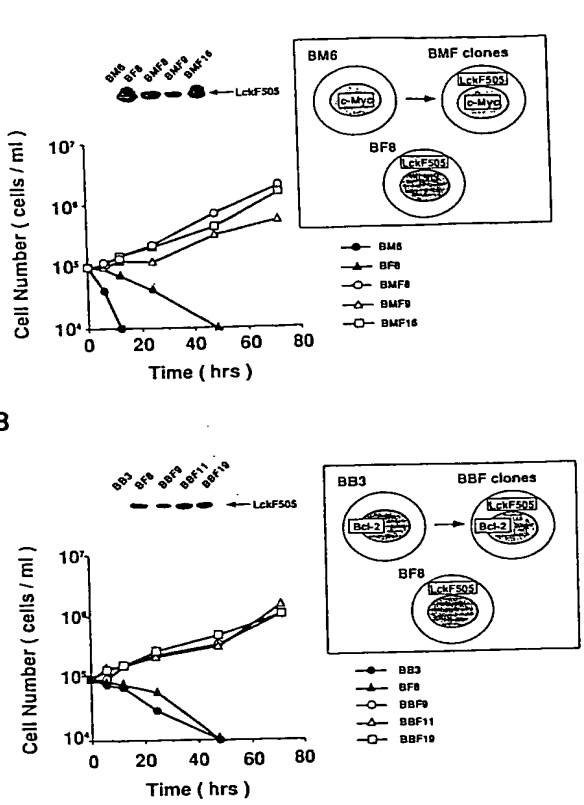


Figure 6. Factor Independency of the BMF and BBF Cells for Cell Growth

Proliferation profiles for the BM6, BF8, BMF8, BMF9, and BMF16 cells (A) and the BB3, BF8, BBF9, BBF11, and BBF19 cells (B) in the absence of growth factor. Expression of p56$^{lck}$F505 was assessed by Western blot analysis and is indicated by the arrows (left inset). Synchronized cells were plated at 1 × $10^5$ cells/ml in the absence of IL-2, IL-3, and EGF. The density of viable cells was counted at various times after plating and represented on a logarithmic scale. The IL-3R and EGFR are not shown in the right inset panel.

(Figure 5B). Next, pdKCR-lckF505 was similarly cotransfected with the *puro* resistance gene into the BAF-B03-derived BM6 and BB3 cells that constitutively express the c-*myc* and *bcl-2* genes, respectively. Transformants expressing p56$^{lck}$F505 were obtained, and these were termed BMF and BBF cells, respectively. Unlike BF cells, both BMF and BBF cells could proliferate in a growth factor–independent manner (Figures 6A and 6B). Supernatants from these cells showed no detectable growth factor activity (data not shown). These observations indicate that p56$^{lck}$ activation per se is insufficient to promote cell proliferation but that it can cooperate with either c-Myc or Bcl-2, in a manner analogous to EGFR stimulation. Taken together, these observations suggest that the IL-2R is linked to at least three pathways leading to the activation of target genes critical for proliferative signal transmission (i.e., the *src* family PTK-linked c-*fos*/c-*jun* induction pathway, the c-*myc* induction pathway, and the RAP-sensitive, *bcl-2* induction pathway).

### Suppression of Apoptosis by p56$^{lck}$

It has been demonstrated that c-Myc induces apoptosis when expressed in the absence of serum or growth factors (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992), whereas Bcl-2 suppresses it (Bissonnette et al., 1992). When BER2, BM6, or BB cells were starved of IL-3 for 15 hr and the number of viable cells subsequently was counted at various intervals, accelerated cell death, accompanied by DNA fragmentation (data not shown), was observed in BM6 cells constitutively expressing the human c-*myc* gene, compared with the parental BER2 cells. In contrast, BB cells expressing the human *bcl-2* gene could survive for a prolonged period relative to BER2 cells (Figure 7A). Interestingly, BF cell lines, which express an active form of p56$^{lck}$, could survive in the absence of growth factors for a prolonged period relative to the parental BER2 cells (Figure 7B), and DNA fragmentation was also sup-

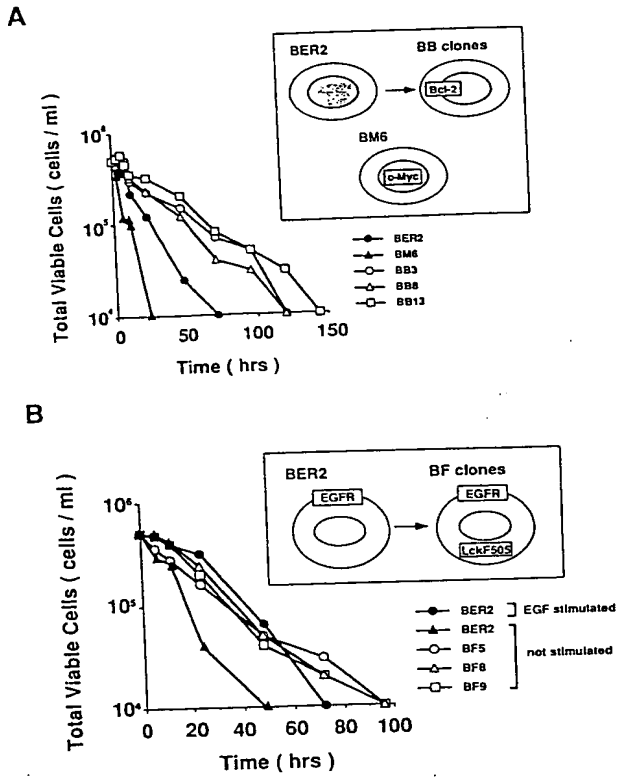


Figure 7. Regulation of Apoptosis in BAF-B03 Derived Cells by c-*myc*, *bcl-2*, or *lck*F505 Genes Expressed Exogenously

(A) Cell viability of BER2, BM6, BB3, BB8, and BB13 cells following factor deprivation. Synchronized cells were plated at 5 × $10^5$ cells/ml in the absence of IL-2, IL-3, and EGF. The density of viable cells was counted at various times after plating and represented on a logarithmic scale.

(B) Cell viability of BF5, BF8, and BF9 cells following factor deprivation and of the parental BER2 cells with or without EGF stimulation. Synchronized cells were plated at 5 × $10^5$ cells/ml with or without EGF stimulation. The number of viable cells was counted at various times after plating. The IL-3R and EGFR are not shown in the right inset panel.

pressed (data not shown). In all BF clones, constitutive expression of an active form of p56$^{lck}$ did not affect *bcl-2* mRNA expression in the absence of growth factors (data not shown). In addition, EGFR stimulation of the parental BER2 cells also resulted in suppression of cell death (Figure 7B). These observations suggest that, like Bcl-2, p56$^{lck}$ activation, as well as EGFR stimulation, results in the suppression of apoptosis.

## Discussion

Many cytokine/cytokine receptor systems have been extensively studied in the context of the regulation of cell proliferation, particularly in hematopoietic lineages. It has been shown that IL-2 induces tyrosine phosphorylation of a number of intracellular proteins and that the IL-2R is coupled both physically and functionally with multiple nonreceptor-type PTKs (see Introduction). Despite the identification of such PTKs as downstream mediators of IL-2 signal transmission, the nature of their target genes and the mechanisms whereby they promote cell cycle progression are not fully understood.

### *bcl-2* as a Critical Target in IL-2 Signaling

In the present study, we have provided evidence that, in addition to c-*fos*/c-*jun* and c-*myc* genes, another proto-oncogene (*bcl-2*) is critical for IL-2 signaling. Analysis of BAF-B03-derived clones expressing IL-2Rβ mutants has revealed that the induction of both the c-*myc* and *bcl-2* genes is mediated by the S region of the IL-2Rβ chain. It is interesting to note, however, that RAP inhibits *bcl-2* gene induction but not c-*myc* gene induction. Hence, our observations suggest the presence of a RAP-sensitive intracellular IL-2 signaling pathway leading to *bcl-2* gene induction and indicate that the induction pathways for *bcl-2* and c-*myc* must diverge at a point beyond that which is mediated by the S region of the IL-2Rβ chain. Recently, a target protein of the RAP–FKBP12 complex, termed FRAP/RAFT1, has been identified that appears to be a mammalian homolog of the yeast TOR proteins (Brown et al., 1994; Sabatini et al., 1994). In view of the fact that both TOR and FRAP/RAFT1 proteins show homology to lipid kinases, one might speculate a link between such kinases and the *bcl-2* induction.

As a result of the finding that IL-2-induced *bcl-2* gene expression is selectively affected by RAP, we established F7-derived transformants that constitutively expressed the *bcl-2* gene and examined the sensitivity of these cells to RAP. These clones proliferated better than the parental F7 cells in the presence of RAP, but remained partially sensitive to RAP (T. M., unpublished data). This may reflect the fact that other biochemical pathways are also RAP sensitive. For example, it is known that RAP inhibits the activation of p70$^{S6K}$ (Chung et al., 1992; Kuo et al., 1992), although at present the relationship between p70$^{S6K}$ activation and *bcl-2* induction by IL-2 is unclear.

Our present findings provide evidence for the importance of the *bcl-2* proto-oncogene in the regulation of the hematopoietic cell cycle. *bcl-2* gene induction is selectively inhibited by RAP, and this correlates with the inhibition of cell cycle progression. The cell cycle progresses when Bcl-2 expression is accompanied by the expression of c-Myc or p56$^{lck}$F505, or by EGFR signaling in BAF-B03-derived cells. Thus far, the function of Bcl-2 has been extensively studied in the context of suppression of apoptosis (reviewed by Korsmeyer, 1992; Schwartz and Osborne, 1993; Collins and Rivas, 1993; Vaux, 1993). In this context, our results may be interpreted to mean that EGFR stimulation or constitutive expression of c-Myc or p56$^{lck}$F505 leads to simultaneous proliferative and apoptotic stimuli and that Bcl-2 suppresses the apoptotic stimulus, resulting in cell proliferation (see Harrington et al., 1994). On the other hand, our results suggest another possibility: Bcl-2 may also play a role in promoting hematopoietic cell proliferation independent of its role in the inhibition of apoptosis. While these two possibilities cannot be rigorously distinguished at present, the following observations argue for the latter possibility: first, although c-Myc

can provide an apoptotic stimulus in BAF cells, both EGFR stimulation and p56$^{lck}$F505 expression, each of which also cooperates with Bcl-2, in fact suppress apoptosis (Figure 7B); second, in BB cells, which express Bcl-2 constitutively, cyclin D3 mRNA, but not the D1 and D2 mRNAs, remains elevated after cytokine deprivation, whereas cyclin D3 expression declines in the parental BER2 cells. In fact, constitutive expression of cyclin D3 along with either c-Myc or p56$^{lck}$F505 expression allows the cytokine-independent proliferation of BAF cells. In contrast, cyclin D3 and Bcl-2 do not cooperate in this manner (Z.-J. L. and T. M., unpublished data). Although further work will be required to elucidate the precise mechanism by which Bcl-2 affects the cell cycle, the results presented here nevertheless provide strong evidence that *bcl-2* induction by IL-2 (or IL-3) is critical for cell cycle progression.

### The Role of p56$^{lck}$ in IL-2-Induced Cell Proliferation

Our results with the active form of p56$^{lck}$ (p56$^{lck}$F505) also suggest a role for this PTK in the transmission of the IL-2-induced mitogenic signal(s). Previously, we had shown that the IL-2Rβ mutant lacking the acidic region (A mutant) fails to associate with and activate the p56$^{lck}$ PTK but still induces cell proliferation (Hatakeyama et al., 1989; Minami et al., 1993). In view of the results presented here, it is most likely that cells expressing the A mutant proliferate in response to IL-2 stimulation via the induction of c-*myc* and *bcl-2* in the absence of p56$^{lck}$ activation. Consistent with this notion is the observation that expression of these two genes in combination can render BAF-B03-derived cells cytokine independent (Figure 5A).

Furthermore, coexpression of p56$^{lck}$F505 with either c-Myc or Bcl-2 also promotes cell cycle progression in the absence of cytokines. p56$^{lck}$F505 expression, however, cannot cooperate with EGF stimulation to promote cell proliferation (Figure 5B). In addition, p56$^{lck}$F505, as well as EGFR stimulation, appears to suppress apoptosis upon IL-3 deprivation in BAF-B03-derived cells (Figure 7B). We infer that the p56$^{lck}$ PTK elicits a signal similar or identical to that of the EGFR (Figure 1). In fact, it has been shown previously that c-*fos*, c-*jun* and their family members can be activated by a p56$^{lck}$-linked pathway or by EGFR signaling in BAF-B03 cells (Shibuya et al., 1992).

### Cooperation of the Three Signaling Pathways

Our results indicate the existence of at least three distinct signaling pathways linked to the IL-2R: the p56$^{lck}$ pathway that leads to c-*fos*/c-*jun* induction, the *bcl-2* induction pathway, and the c-*myc* induction pathway (Figure 1). Importantly, none of these pathways affects the activation of the other: c-*myc* gene expression does not affect the induction of c-*fos*/c-*jun* or *bcl-2*, *bcl-2* gene expression does not affect c-*fos*/c-*jun* or c-*myc* expression, and p56$^{lck}$ PTK activation does not induce the *bcl-2* or c-*myc* genes (Figures 2 and 4A; T. M., unpublished data).

Expression studies show that a combination of any two of the three pathways is sufficient to promote the growth of BAF-B03 cells in the absence of cytokines (Figures 5A, 6A, and 6B). We infer that these three pathways cooperate with each other to ensure a full-scale signal transmission by IL-2. Consistent with this are the observations that F7 cells expressing wild-type IL-2Rβ respond to IL-2 better than A15 cells that express the IL-2Rβ A mutant (Shibuya et al., 1992) and that BMB cells that are able to proliferate in the absence of cytokines via the constitutive expression of c-*myc* and *bcl-2* can also respond to EGF as indicated by an apparent further increase in the rate of proliferation (approximately 1.5-fold; T. M., unpublished data).

The induction pathways of both the c-*myc* and *bcl-2* genes are linked to the S region of the IL-2Rβ chain. Recently, it was found that this region of IL-2Rβ is necessary for the binding of Jak1 (Miyazaki et al., 1994; Witthuhn et al., 1994) and Syk PTK (Minami et al., 1995) and that the carboxy-terminal region of IL-2Rγ is necessary for association with Jak3 (Johnston et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). It was also shown that the activation of Syk PTK results in the induction of the c-*myc* gene (Minami et al., 1995; Figure 1). Thus, it is likely that Jak1, Jak3, and Syk are involved in the induction of the above proto-oncogenes by IL-2. More recently, we have found that IL-2 activates Stat5 in F7 cells. This Stat5 activation (presumably by the IL-2R-associated Jak PTKs) requires the membrane distal H region, yet this region is dispensable for IL-2-induced cell proliferation (H. Fujii and T. T., unpublished data; Figure 1; Hatakeyama et al., 1989). Therefore, it is unlikely that Stat5 activation is involved in the induction of these proto-oncogenes.

Although in our experimental system activation of two out of three pathways was required for cell proliferation, it is possible that activation of all three pathways may be required for the proliferation of certain cells, e.g., peripheral T cells, depending on the magnitude of induction of these target genes. In this regard, it is important to note that our inferences have certain limitations, since they are primarily based on the results of constitutive gene expression assays, whereas the induction of these genes is normally of a transient nature. The question of how the signaling mechanisms operating in BAF-B03 cells relate to other cells, such as peripheral T cells, also remains to be addressed. Nevertheless, in view of the findings that IL-2-induced activation of *src* family PTKs (the Syk PTK, Jak1 and Jak3 PTKs) is observed and that all of the target genes (c-*fos*/c-*jun*, c-*myc*, and *bcl-2*) are also induced by IL-2, in both normal lymphocytes and BAF-B03 derived cells, we think that BAF-B03 cells retain similar, if not identical, mechanisms of cell proliferation. The fact that RAP, which selectively inhibits the *bcl-2* gene induction pathway, inhibits the growth of T cells (Sigal and Dumont, 1992) and BAF-B03 cells (data not shown) is also consistent with this view. At the same time, our results do not exclude the possibility of additional IL-2R-linked signaling pathways.

## Experimental Procedures

### Cells and Cell Culture

BAF-B03, a subclone of BAF3 cell line, is a bone marrow–derived murine IL-3-dependent pro-B cell line (Palacios and Steinmetz, 1985; Collins et al., 1992). F7 is a BAF-B03-derived stable transformant clone expressing the wild-type human IL-2Rβ chain. A15 and S25 are also BAF-B03-derived stable transformant clones expressing the human

IL-2Rβ mutant lacking the cytoplasmic "acidic" region and the "serine-rich" region, respectively (Hatakeyama et al., 1989). BER2 is a BAF-B03-derived stable transformant clone that expresses the human EGFR. BM6 is a BER2-derived stable transformant clone expressing the human *c-myc* gene ectopically (Shibuya et al., 1992). BB cells were established by transfecting the human *bcl-2* expression plasmid pSVBT (containing the cDNA for human *bcl-2* gene; Tsujimoto and Croce, 1986) into BER2 cells. BF cells were obtained by transfecting the mouse mutant $p56^{lck}$ expression plasmid pdKCRIckF505 into BER2 cells. BMB and BMF cells were obtained by transfecting the plasmids pSVBT and pdKCRIckF505 into BM6 cells, respectively. BBF cells were obtained by transfecting the plasmids pdKCRIckF505 into BB3 cells. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 10% (v/v) conditioned medium from the WEHI-3B cell line (10% WEHI-conditioned medium) as a source of IL-3.

For the analyses of gene expression, cell growth, and cell viability, cells were stimulated with either WEHI-conditioned medium (at a final concentration of 10% [v/v]) or recombinant human EGF (at a final concentration of 10 ng/ml), after cells were synchronized in G1 phase by depriving them of cytokines for 15 hr (the cell viability after cytokine deprivation was more than 90%).

### DNA Transfection

Plasmid DNAs were transfected into the cells by an electroporation procedure as described previously (Doi et al., 1989). Selection was initiated 24 hr after transfection, using 1 mg/ml hygromycin for BB or BF cells and 7.5 μg/ml puromycin for BMB, BMF, or BBF cells. Drug-resistant colonies were picked up and subsequently cloned by limiting dilution as described previously (Miyazaki et al., 1991).

### Preparation of Probe DNA

The probe DNAs for *c-myc*, *c-fos*, and *c-jun* were prepared as described previously (Shibuya et al., 1992), and the probe for human and mouse *bcl-2* were prepared as follows: human *bcl-2*, 1.0 kb XhoI fragment from the plasmid pSVBT; mouse *bcl-2*, 2.5 kb HindIII fragment from the plasmid pmbcl3.3 (Negrini et al., 1987). The probe for *bax* was a PCR fragment (nucleotides 215–569) amplified from a murine spleen cDNA library. The probe for *bcl-*$x_L$ was the 0.8 kb EcoRI fragment from the plasmid pBS-bcl-$x_L$ (Boise et al., 1993).

### RNA Extraction and Northern Blot Analysis

Total cellular RNA from each cell was prepared as described previously (Hatakeyama et al., 1989). For RNA blot analysis, 10 μg of total RNA was loaded on each slot (Shibuya et al., 1992).

### Western Blot Analysis

Cells ($2 \times 10^6$) were harvested and subjected to the analysis as described previously (Minami et al., 1993). The primary antibodies are rabbit anti-$p56^{lck}$ antiserum 195.7 or anti-human Bcl-2 monoclonal antibody /100.

### Cell Growth Assay and Viability Assay

For the cell growth assay, factor-starved cells were cultured at a density of $1 \times 10^5$ cells/ml in RPMI-1640 supplemented with 10% FCS containing either human EGF (10 ng/ml) or human IL-2 (2 nM) or WEHI-conditioned medium (10% [v/v]). Culture media were changed every 2 days (Miyazaki et al., 1991). For the cell viability assay, factor-starved cells were cultured in PRMI-1640 supplemented with 10% FCS without EGF or cytokines. In both assays, viable cell numbers were determined by trypan blue exclusion assay.

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**Note Added in Proof**

The work on Stat5 that is cited as unpublished data by H. Fujii and T. T. in the Discussion is now in press: Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J. N., Minami, Y., Miyazaki, T., and Taniguchi, T. (1995). Activation of Stat5 by IL-2 requires a carboxyl-terminal region of the IL-2 receptor β chain but is not essential for the proliferative signal transmission. Proc. Natl. Acad. Sci. USA, in press.

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